

The Effects of Macrophage Migration Inhibitory Factor on Antioxidant Responses During Cigarette Smoke Exposure

By

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Dissertation Abstract

Chronic obstructive pulmonary disease (COPD) is the third leading cause of death in the United States. Cigarette smoke (CS) is the leading cause of emphysema, a component of COPD, which is characterized by airway inflammation and destruction of alveolar sacs and gas exchange impairment. The pathogenesis of emphysema remains to be elucidated, but CS-induced endothelial cell (EC) apoptosis is an established mechanism that is both necessary and sufficient to cause emphysematous remodeling. Fallica et al have previously shown that expression of the cytokine macrophage migration inhibitory factor (MIF) is altered in patients with COPD and is significantly attenuated in advanced stage COPD. Preclinical data reveals that chronic CS exposure antagonizes MIF gene expression *in vivo*. Furthermore, MIF deficient mice have increased ROS production, DNA damage and exacerbated emphysematous tissue remodeling. Damico et al have also previously shown that EC apoptosis is enhanced in the absence MIF in the context of CS. Taken together, these provide evidence for MIF's cytoprotective role in the context of CS-induced oxidative stress. MIF has also been shown to antagonize oxidant-mediated injury in other models, which may be linked to its capacity to impact the antioxidant regulator and transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2). The goal of this thesis is to elucidate the mechanisms by which MIF is impacting antioxidant responses during CS-induced oxidative stress.

Our results reported here demonstrate that MIF is a necessary mediator of Nrf2-dependent transcription *in vivo* in a model of CS-induced oxidative injury and emphysematous remodeling. We then demonstrated in primary EC *in vitro* that exogenous MIF was sufficient to drive antioxidant response element (ARE)-driven

transcriptional activity and stabilize Nrf2 protein via a proteasomal-dependent mechanism. There are two recognized regulators of Nrf2 stability. The Kelch-like ECH-associated protein 1 (KEAP1)-Cullin3 (CUL3) ligase complex dynamically regulates Nrf2 by targeting it for proteasomal-mediated degradation under basal conditions. In contrast, DJ-1 has been demonstrated to directly and indirectly promote Nrf2 stability in select models. Given that MIF capacity to interfere with Nrf2's proteasomal-mediated degradation, we sought to understand MIF's effects on the members and regulators of the KEAP1-CUL3 ligase complex, as well as DJ-1.

The CUL3 component of the KEAP1-CUL3 complex is regulated by two established mechanisms 1) post-translational modification (PTM) of CUL3 and 2) via inhibitory protein-protein interactions with CAND1. The recognized PTM of CUL3 involves the cyclic addition and removal of a small ubiquitin-like molecule, NEDD8, through a process known as neddylation and deneddylation, respectively. We found that chronic CS exposure *in vivo* was associated with increased CUL3 neddylation in the lung. This observation is lost in MIF deficient animals exposed to CS, implicating MIF as a regulator of CUL3 PTM in the context of CS-induced oxidative injury. To test the hypothesis that the observed CUL3 neddylation status was sufficient to alter Nrf2 activity, we altered the PTM of CUL3 by ectopic expression of the neddyase, UBC12. This was sufficient to enhance CUL3 neddylation and, importantly, drive Nrf2 expression and ARE activity, supporting a model in which MIF stabilizes Nrf2 in the context of CS *in vivo* via alterations in CUL3 neddylation status. MIF however, was neither necessary nor sufficient to alter CUL3 neddylation *in vitro*, suggesting that the mechanism was indirect or contextual. We then investigated the effects of MIF on the known CUL3

inhibitor, CAND1 and determined that MIF is a novel positive regulator of CAND1 expression. Further, over expression of CAND1 was sufficient to stabilized Nrf2 in ECs *in vitro*.

In addition to negative Nrf2 regulators, we also explored MIF's effects on the DJ-1. DJ-1 is a redox-sensitive molecule that is important in mediating antioxidant responses and antagonizing ROS in neuronal cells. We tested the hypothesis that MIF-mediated Nrf2 stability via a DJ-1-dependent mechanism. Despite reports of DJ-1 regulating Nrf2 in pulmonary epithelial cells (EpiCs) *in vitro*, we found that DJ-1 was not sufficient for Nrf2 stabilization in EC *in vitro*, suggesting that the molecular mechanisms of Nrf2 stabilization may differ between pulmonary EC and EpiCs. In conclusion, we provide evidence that MIF's ability to enhance Nrf2 stability as a novel positive regulator of CAND1, and inhibitor of the KEAP1- CUL3 ligase complex.

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Table of Contents

DISSERTATION ABSTRACT	II
ACKNOWLEDGEMENTS	VI
TABLE OF CONTENTS	VIII
LIST OF FIGURES	XI
ABBREVIATIONS	XII
CHAPTER 1	1
GENERAL INTRODUCTION	1
1. CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD)	2
1.1 DEFINITION	2
1.2 EMPHYSEMA DEFINITION	3
1.3 EPIDEMIOLOGY & GLOBAL BURDEN OF DISEASE	4
1.4 CAUSES & RISK FACTORS	5
1.5 CELLULAR AND MOLECULAR EVENTS IN THE PATHOGENESIS OF EMPHYSEMA	9
1.6 CELLULAR APOPTOSIS & EMPHYSEMA PATHOGENESIS	11
2. MACROPHAGE MIGRATION INHIBITORY FACTOR (MIF)	16
2.1 STRUCTURE & FUNCTION	16
2.2 MIF, APOPTOSIS & EMPHYSEMATOUS REMODELING	21
2.3 THE EMERGING ROLE OF MIF AS A POSITIVE REGULATOR OF ANTIOXIDANT RESPONSES	23
3. OXIDANTS AND ANTIOXIDANT RESPONSES	25
3.1 OXIDATIVE STRESS, ANTIOXIDANTS	25
3.2 ANTAGONISM OF ROS EFFECTS BY THE ANTIOXIDANT RESPONSE ELEMENT (ARE)	28
4. NUCLEAR FACTOR (ERYTHROID-DERIVED)–LIKE 2 (NRF2)	29
4.1 DISCOVERY OF NRF2	29
4.2 NRF2 STRUCTURE: THE SEVEN DOMAINS OF NRF2	30
4.3 REGULATION OF NRF2	33
5. CULLIN E3 RING LIGASES & THEIR ROLE IN THE POLYUBIQUINATION OF NRF2	40
5.1 THE ROLE OF CRL3 SUBCLASS IN NRF2 REGULATION	41
5.2 REGULATION OF CRL'S	42
6. DJ-1 & REGULATION OF ANTIOXIDANT RESPONSES	47
6.1 STRUCTURE OF DJ-1	47
6.2 THE CYTOPROTECTIVE ROLES OF DJ-1	49
CHAPTER 2	52
METHODS & MATERIALS	52
2.1 EXPERIMENTAL MODELS	53
2.1.1 ANIMAL MODELS	53
2.2 RNA ISOLATION & RT-PCR	54
2.3 WESTERN BLOT	55
2.4 PLASMID PREPARATION & ISOLATION	55
2.5 TRANSFECTIONS	56
2.6 DUAL LUCIFERASE ASSAY	57

2.7 CASPASE 3 ACTIVITY ASSAY	57
2.8 STATISTICAL ANALYSIS	58
CHAPTER 3	59
THE EFFECTS OF MIF ON THE NRF2-ANTIOXIDANT RESPONSE PATHWAY	59
3.1 ABSTRACT	60
3.2 INTRODUCTION	62
3.3 RESULTS	67
3.3.1 MIF IS NECESSARY FOR CS-INDUCED ANTIOXIDANT TRANSCRIPTS IN THE LUNG <i>IN VIVO</i>	67
3.3.2 RECOMBINANT MIF (RMIF) DECREASES CS-INDUCED ROS ACCUMULATION <i>IN VITRO</i>	68
3.3.3 MIF DRIVES ARE ACTIVITY IN PULMONARY ECS	69
3.3.4 MIF IS NECESSARY FOR OPTIMAL ARE ACTIVITY UNDER CONDITIONS OF OXIDATIVE STRESS	70
3.3.5 RMIF PROMOTES STABILIZATION OF NRF2 IN A PROTEASOME-DEPENDENT MANNER	71
3.4 DISCUSSION & CONCLUSION	73
CHAPTER 4	86
MIF INHIBITS PROTEASOMAL-MEDIATED DEGRADATION OF NRF2 VIA UPREGULATION OF CAND1	86
4.1 ABSTRACT	87
4.2 INTRODUCTION	89
4.3 RESULTS	92
4.3.1 MIF DOES NOT EFFECT KEAP1 EXPRESSION <i>IN VIVO</i> AND <i>IN VITRO</i>	92
4.3.2 CHRONIC CS EXPOSURE ALTERS CUL3 NEDDYLYATION: IMPACT OF MIF DEFICIENCY	94
4.3.3 CHRONIC CS EXPOSURE INDUCES EXPRESSION OF THE DENEDDYLYASE, JAB1, IN THE ABSENCE OF MIF	95
4.3.4 EXOGENOUS MIF DOES NOT ALTER EXPRESSION OF TOTAL CUL3 OR NEDD8-CUL3 <i>IN VITRO</i>	95
4.3.5 OVEREXPRESSION OF THE NEDDYLYASE, UBC12 INCREASES NEDD8-CUL3	96
4.3.6 UBC12 OVEREXPRESSION INDUCES NRF2 EXPRESSION <i>IN VITRO</i>	97
4.3.7 JAB1 DOES NOT AFFECT THE NEDDYLYATION OF CUL3 <i>IN VITRO</i>	97
4.3.8 FORCED EXPRESSION OF DOMINANT NEGATIVE UBC12 DOES NOT ALTER CUL3 NEDDYLYATION <i>IN VITRO</i>	99
4.3.9 MIF DOES NOT ALTER JAB1 EXPRESSION <i>IN VITRO</i>	99
4.3.10 EXOGENOUS MIF INCREASES THE EXPRESSION OF THE CRL INHIBITOR, CAND1	100
4.3.11 CAND1 OVEREXPRESSION INCREASES NRF2 PROTEIN	100
4.4 DISCUSSION & CONCLUSION	101
CHAPTER 5	119
ROLE OF DJ-1 IN PULMONARY ENDOTHELIAL CELLS	119
5.1 ABSTRACT	120
5.2 INTRODUCTION	122
5.3 RESULTS	124
5.3.1 DJ-1 IS DIFFERENTIALLY EXPRESSED IN THE LUNG TISSUES OF WILDTYPE AND MIF DEFICIENT MICE	125
5.3.2 THE LOSS OF DJ-1 DOES NOT DOWNREGULATE MIF EXPRESSION	125
5.3.3 DJ-1 EXPRESSION IS NOT ALTERED <i>IN VITRO</i> IN RESPONSE TO OXIDATIVE STRESS	126

5.3.5 DJ-1 IS NOT SUFFICIENT TO INCREASE NRF2 EXPRESSION OR ACTIVITY <i>IN VITRO</i>	127
5.3.6 DJ-1 DEFICIENCY DOES NOT SENSITIZE ECs TO THE EFFECTS OF CSE	128
5.4 DISCUSSION & CONCLUSION	129
<u>CHAPTER 6</u>	<u>138</u>
<u>THESIS SUMMARY</u>	<u>138</u>
6.1 SUMMARY OF THESIS	139
6.2 LIMITATIONS OF WORK	145
6.2.1 LIMITATIONS OF IN VITRO AND IN VIVO MODELS	146
6.2.2. TECHNICAL LIMITATIONS	147
6.3 IMPLICATIONS OF FINDINGS AND FUTURE DIRECTIONS	148
6.4 CONCLUSION	149
<u>REFERENCE</u>	<u>150</u>
<u>CURRICULUM VITAE</u>	<u>182</u>

List of Figures

Chapter 3

Figure 3-1	MIF is necessary for CS-induced antioxidant transcription <i>in vivo</i>	80
Figure 3-2	rMIF decreases CS-induced ROS accumulation <i>in vitro</i>	81
Figure 3-3	rMIF induces ARE-activity <i>in vitro</i>	82
Figure 3-4	MIF loss attenuates CS-induced ARE activity	83
Figure 3-5	MIF-induced Nrf2 expression in ECs is proteasome-dependent	85

Chapter 4

Figure 4-1	rMIF does not effect KEAP1 expression <i>in vitro</i>	108
Figure 4-2	MIF-dependent CUL3 neddylation <i>in vivo</i>	109
Figure 4-3	MIF-deficiency increases CS-induced JAB1 expression in the intact murine lung	110
Figure 4-4	rMIF does not alter CUL3 neddylation <i>in vitro</i>	111
Figure 4-5	UBC12-mediated CUL3 neddylation	112
Figure 4-6	UBC12-driven Nrf2 protein expression and ARE activity	113
Figure 4-7	JAB1 overexpression does not alter CUL3 neddylation pulmonary ECs	114
Figure 4-8	JAB1 deficiency does not alter the neddylation of CUL3	115
Figure 4-9	dnUBC12 expression does not alter CUL3 neddylation	116

Figure 4-10	rMIF does not alter JAB1 expression <i>in vitro</i>	117
Figure 4-11	MIF-induced CAND1 expression	118
Figure 4-12	CAND1-mediated Nrf2 expression	119

Chapter 5

Figure 5-1	DJ-1 is upregulated in lung of MIF^{-/-} Mice	134
Figure 5-2	The loss of DJ-1 does not impact MIF protein expression	135
Figure 5-3	H₂O₂ does not alter DJ-1 expression in pulmonary ECs	136
Figure 5-4	DJ-1 overexpression does not alter Nrf2 expression or ARE activity in EC <i>in vitro</i>	137
Figure 5-5	CS-Mediated Caspase 3 activity	138

Abbreviations

AHR	Aryl hydrocarbon receptor
AP-1	Activated Protein-1
ARE	Antioxidant Response Element
BAD	BCL2-associated agonist of cell death
BAL	Bronchoalveolar lavage fluid
BRG	Brahma-related gene-1
BTB	Tramtrack and bric-a-brac
Bzip	Basic-region leucine zipper
CAND1	Cullin-Associated NEDD8-Disassociated Protein 1
CBP	(cAMP Responsive Element Binding protein) Binding Protein
CHD6	Chromodomain helicase DNA-binding protein 6
CREB	cAMP Responsive Element Binding protein
COPD	Chronic Obstructive Pulmonary Disease
CRL3	Cullin RING Ligase Complex 3
CNC	Cap'n'collar
CS	Cigarette Smoke
CSE	Cigarette Smoke Extract
CUL3	Cullin 3
CTD	C-terminal domain
DAXX	Death domain associated protein
DCFDA	2',7'-Dichlorofluorescein Diacetate
DCF	Dichlorofluorescein
DCN	Defective in cullin neddylation 1 protein
DEN1	Deneddylase 1
dnUBC12	Dominant Negative UBC12
EC	Endothelial Cells
EpiC	Epithelial Pneumocytes
FEV1	Forced Expiratory Volume in 1 Second
FLIP	FLICE-like inhibitor protein
FVC	Forced Vital Capacity
HECT	Homologous to E6-AP Carboxy Terminus
HMVEC	Human Lung Microvascular Endothelial Cells
GOLD	Global Initiative for Chronic Obstructive Lung Disease
GPX2	Glutathione Peroxidase 2
IL-8	Interleukin-8
JAB1	c-Jun Activation Domain Binding Protein-1
JAMM	JAB1 MPN Domain Metalloenzyme Motif
JNK	Jun-N-terminal Kinase
KEAP1	Kelch-like ECH-associated protein 1
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinases
MDA	Malondialdehyde
MAF	Small masculoaponeurotic fibrosarcoma
MIF	Macrophage Migration Inhibitory Factor

MIF ^{-/-}	MIF Deficient mice
MIF ^{+/+}	Wild-type Mice
NAC	N-acetyl cysteine
NAE	NEDD8 E1 activating enzyme
NEDD8	Neural Precursor Cell Expressed, Developmentally Down-Regulated 8
Neh	Nrf2_ECH homology
NF-κB	Nuclear factor light-chain-enhance of activated B-cell
NQO1	NADPH dehydrogenase quinone 1
NRF2	Nuclear factor erythroid 2-related factor 2
NTD	N-terminal domain
OT	On-Target
p450s	Cytochrome P450
p53	Transformation-related protein 53
RILI	Radiation-induced lung injury
RING	Really Interesting New Genes
rMIF	Recombinant MIF
RMVEC	Rat Pulmonary Microvascular Endothelial Cells
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
PD	Parkinson's Disease
POZ	Poxvirus and zinc finger
PTEN	Phosphatase and tensin homolog
SCF	Skp1-Cullin1-F-box
SOD	Superoxide dismutase
TBHQ	Tert-Butylhydroquinone
TCDD	2,3,7,8-tetrachlorodibenzo- <i>p</i> -dioxin
TLR	Toll-like receptors
TNF	Tumor necrosis factor
TPOR	Thiol-Protein Oxidoreductases
TRX	Thioreoxin
VEGF	Vascular endothelial growth factor
Veh	Vehicle
UBC12	Ubiquitin-conjugating enzyme E2M
XOR	Xanthine Oxidoreducase
XRE	Xenbiotic response element

Chapter 1

General Introduction

1. Chronic Obstructive Pulmonary Disease (COPD)

1.1 Definition

Chronic obstructive pulmonary disease (COPD) is a progressive and largely irreversible disease that remains to be a major public health problem. It is a heterogeneous disease with many comorbidities that is comprised of chronic bronchitis, emphysema and non-reversible asthma (Izumi 2003). COPD is a term that describes a group of pulmonary diseases, characterized by slow progression of flow airflow obstruction, gas exchange impairment, air trapping and mucus hyper-secretion (Hogg and Timens 2009). Emphysema and chronic bronchitis are the two main types of COPD. Chronic bronchitis is defined as the presence of a productive cough on most days for three months in two consecutive years independent of other causes of chronic cough such as tuberculosis and heart failure. The pathological features of chronic bronchitis include, goblet cell hyperplasia, inflammation and hypertrophy, which ultimately lead to fibrosis of the upper airways (Hoidal 1994, Nelson and Mason 2000). Emphysema is defined histologically and is characterized by progressive and irreversible enlargement of airspace due to the destruction of alveolar sacs (reviewed in (Fischer, Pavlisko et al. 2011). Patients can exhibit a mix of different characteristic features of both emphysema and chronic bronchitis, but ultimately all display airway obstruction.

The Global Initiative for Chronic Obstructive Lung Disease (GOLD) was launched in 1997 to raise awareness about chronic COPD and set a criterion for classification. It utilizes evidence-based documents to set guidelines for detection, treatment and management. According to GOLD, COPD is defined as the following:

“a preventable and treatable disease characterized by persistent airflow limitation that is usually progressive and associated with enhanced chronic inflammatory response in the airways and the lung to hazardous particles and gases. Exacerbations and comorbidities contribute to the severity in individual patients.”

The GOLD criteria have been established as a way of classifying disease severity base on airflow limitation. The GOLD criteria use spirometry as an assessment of pulmonary function. It classifies disease severity as a ratio of forced expiratory volume in 1 second (FEV₁) to forced vital capacity (FVC). An FEV₁/FVC of less than 0.7 indicates airway obstruction. The GOLD definition has been adopted by both, the American Thoracic Society (ATS) as well as the European Respiratory Society (ERS) (Holman, 1966).

1.2 Emphysema Definition

Emphysema is a component of COPD that is characterized by the permanent enlargement of the air spaces distal to the terminal bronchioles due to the progressive destruction of the alveolar walls as a consequence of damage to the connective tissue, ultimately leading to impaired gas exchange (reviewed in (Cantin and Crystal, 1985)). It is a disease of the distal to terminal bronchioles that is defined as: *“a condition of the lung characterized by permanent abnormal enlargement of the respiratory airspaces, accompanied by destruction of their walls without obvious fibrosis”* (American Thoracic Society, 1962).

1.3 Epidemiology & Global Burden of Disease

The epidemiology of COPD has been difficult to characterize due to disease heterogeneity and misclassification. It is established that COPD is one of the leading causes of death in both low and high-income countries (Celli, MacNee et al. 2004). According to the National Institute of Health, COPD was the third leading cause of death in 2014 (Mannino and Buist 2007) and estimated 15.7 million (6.4%) Americans have reported being diagnosed with it (Raherison and Girodet 2009). It is also the fifth leading cause of years lost to mortality or disability-adjusted life years. The estimated prevalence of COPD in the US, based on spirometry (FEV1<60-65% of predicted value) was about 4–6% and 1–3% of adult white males and females, respectively (National Center for Health Statistics, (2016)). The prevalence of COPD is double in males as compared to females, it is expected that this discrepancy will diminish. This is due to the fact that more women have taken up smoking in developed countries. An increase in biomass exposure in non-smoking women will also reduce the gender-driven differences of COPD prevalence (Mannino and Buist 2007). The World Health Organization estimated that COPD accounted for about 30 million deaths, globally and 3 million deaths in North America. The estimated prevalence of COPD is 5-15% (Higgins and Thom, 1989).

There are many comorbidities associated with COPD that impact multiple organ systems, such as the cardiovascular and musculoskeletal system, which further complicate disease management and drive economic burden (Barnes and Celli 2009, Barnes, Calverley et al. 2013). Currently, management and treatment of COPD is about \$36 billion, with projected directed costs being about \$49 billion by 2020. Furthermore, COPD contributed to about 16.4 million workdays costing about \$3.9 billion lost in

absenteeism in 2014 (Center for Disease Control, 2014 (Murray and Lopez 1997)). In conclusion, COPD is a costly disease that accounts for increased health expenses and time lost in the workplace.

1.4 Causes & Risk Factors

While smoking remains to be the number one risk factor for COPD, as it contributes to about 80-90% of COPD in the US (US Department of Health and Human Services, 2002), the presence of disease in non/never smokers indicates other risk factors that are responsible for disease manifestation. COPD is a multifactorial disease that is the result of interplay between genetics and environmental factors (Eisner, Anthonisen et al 2011).

1.3.1 Environmental Risk Factors

Chronic exposure to CS remains the number one risk factor for developing emphysema. Cigarettes are comprised of 600 ingredients that release complex mixture of over 4,000 chemicals when burned to produce CS (Department of Health and Human Services, 1964). CS is a heterogeneous and potent source of oxidants that represent about twenty-two different classes of compounds (Borgerding and Klus 2005). It is comprised of a particulate phase, which is condensed liquid particles suspended in a volatile/semi-volatile gaseous phase (Church and Pryor 1985). Each puff of mainstream CS results in the inhalation of about 10^{15} free radicals (Valavanidis, Vlachogianni et al. 2009). The exact mechanism by which CS induces lung damage is still unclear, but its capacity to promote chronic inflammation has been well established as a major contributor in COPD

pathogenesis (Macnee 2007). Incidences of COPD in never-smoked individuals provide evidence for other risk factors that play a role the development of disease (Zeng, Sun et al. 2012).

According to the Third National Health and Nutrition Examination Survey, never smokers represented 23% of burden of disease in the US (Celli, Halbert et al. 2005). The effects of first-hand smoking on the development of disease differ according to regions and studies. The range by which smoking serves as the major contributor of COPD is 9.7-97.9%, which provides evidence for the effects of environmental and other risk factors (reviewed in (Eisner, Anthonisen et al. 2010)). There is a lot of evidence of environmental exposures serving as risk factors for COPD development and it is not a new concept. Phillips first identified the role of occupational and industrial exposure in chronic bronchitis in 1963. He not only shed light on the effects of air pollution on bronchitis exacerbation, but he also introduced the idea that environmental exposures can be a *cause* of respiratory disease (Phillips 1963). Other environmental causes of COPD independent of smoking include: indoor air pollution (Salvi and Barnes, 2010 (Gunen, Hacievliyagil et al. 2008), outdoor air pollution (Kunzli, Kaiser et al. 2000, Schikowski, Sugiri et al. 2005), socioeconomic status and chronic asthma (Husman, Koskenvuo et al. 1987, Kogevinas, Anto et al. 1998).

1.3.2 Genetic Risk Factors

Genetics plays an important role in the development of COPD. While smoking is the leading cause of COPD, only about 15-20% of smokers develop disease, which suggests that there are differences in susceptibility which are genetic in nature

(Louhelainen, Ryttilä et al. 2009). Larson et al showed that COPD tends to aggregate in families (Larson, Barman et al. 1970). Furthermore, a monozygotic twin study had shown the potential influence of genetics on pulmonary function (Man and Zamel 1976). While linkage studies show aggregation of the disease among families, they are limited in their capacity to separate genetics from shared familial environments. The genes that play a role in the development of COPD are ones that affect the proteases and anti-proteases, inflammation, metabolism of CS-derived toxins as well as the mucociliary clearance system in the lung.

An established gene, whose mutation or deficiency does contribute to the manifestation of COPD is the SERPINA1 gene, which encodes for alpha-1 antitrypsin (AAT) (Webb, Hyde et al. 1973), an anti-protease. Imbalance of proteases and anti-proteases drives parenchymal lung damage, ultimately resulting in emphysematous remodeling. SERPINA1 is activated under conditions of oxidative stress. Laurell and Eriksson first identified the protein in an electrophoresis experiment in 1963 and noted that its loss is associated with early-onset COPD (Laurell and Erickson, 1963, American Thoracic Society/European Respiratory Society, 2003). AAT is a glycoprotein (52 kDal) that is secreted by many different cell types (Geboes, Ray et al. 1982). Once secreted, it goes into circulation where it can inhibit a number of proteases, including excess neutrophil elastases, pancreatic trypsin, granzyme-B and many other circulating proteases (Bergin, Reeves et al. 2010):

In addition to pulmonary tissue remodeling, the effective metabolism of noxious chemicals, like those derived from CS or air pollution is an important mechanism that protects the lungs from CS-induced. Furthermore, COPD is also the result of an

imbalance of oxidants and antioxidants that lead to alterations of the redox status in the lung. Therefore, compromised or inefficient xenobiotic metabolism can lead to increased ROS production and oxidative injury (reviewed in (MacNee 2000, Sandford and Pare 2000)). Inherited deficiencies in the xenobiotic metabolizing enzymes such as, glutathione S-transferases (GSTs) (Ishii, Matsuse et al. 1999) and cytochrome p450s (Cantlay, Lamb et al. 1995) can increase individual susceptibility to the development of COPD. These enzymes are critical in remediating the effects of free radicals and electrophilic compounds, such as those found in CS (Sheehan, Meade et al. 2001). GSTs are highly polymorphic therefore their detoxifying capacity is heavily dependent on the inherited genotype. *Dey et al.* found that the GSTM1 null genotype is significantly linked to COPD susceptibility and demonstrated that it is a risk factor for smokers who live in close proximity to coal mines (Dey, Gogoi et al. 2014). Other drug metabolizing enzymes that can potentially influence the susceptibility of COPD are cytochrome p450 family of enzymes. Like GSTs, these enzymes are highly polymorphic and a meta-analysis by Wang et al., found that polymorphic variants of CYP1A1 may contribute to differences in COPD susceptibility (Wang, Chen et al. 2015). These provide evidence for the importance of roles of genetic polymorphism in modulating individual susceptibility to the development of COPD.

Ultimately, only a fraction of smokers develop COPD. This indicates that genetic polymorphisms play an important role in modulating an individual's risk in developing disease. Genetic variation in the activities and expression of genes that play roles in oxidative stress, detoxification, inflammation and remodeling are important in understanding disease susceptibility.

1.5 Cellular and Molecular Events in the Pathogenesis of Emphysema

Emphysema is a complex, multifactorial disease. Pathologically, it caused by two major processes, narrowing and remodeling of the small airways and destruction of the lung parenchyma with subsequent alveolar destruction (Hogg, Chu et al. 2004). Chronic exposures to sources of oxidative stress like those found in cigarette smoke and environmental pollutants can initiate disease, which is then perpetuated by abnormal immune responses, alterations in lung tissue repair and attenuated antioxidant responses. There are three cellular and molecular events that form a triad, which drives disease pathogenesis. These include increased oxidative stress, which causes an imbalance between oxidants and antioxidants, chronic inflammation and protease/anti-protease imbalance. These events are all inter-related and interconnected as they can all cause and promote one another. Collectively, they lead to increased apoptosis, which perpetuates disease in the context of emphysema. This dissertation will focus on the effects of oxidative stress and the ultimate effects on cell apoptosis as driver for emphysematous remodeling.

1.5.1 Oxidative Stress and Initiation of Disease

Oxidant toxicity in the lung is the result of prolonged exposure to noxious chemicals such as those found in cigarette smoke and other environmental exposures that perpetuate the state of increased oxidative stress. There is mounting evidence that markers of oxidative stress are elevated in patients with COPD as compared to their healthy counterparts. These markers of oxidative stress have been characterized in the

setting of increased oxidative burden among smokers that can be obtained from blood, sputum and bronchoalveolar lavage fluid (BAL). These include markers of DNA damage, concentration of oxidized proteins, lipid peroxidation and generation of ROS (Liu, Sandrini et al. 2007). Exhaled breath condensate is another method employed to assess the concentrations of limited free radicals such as reactive nitrogen species (RNS), hydrogen peroxide and 8-isprostane can be observed in exhaled breath condensate (EBC) of individuals with COPD (Dekhuijzen, Aben et al. 1996, Montuschi, Collins et al. 2000, Corradi, Montuschi et al. 2001).

COPD initiation (and progression) is the result of chronic exposure to oxidants that overwhelm antioxidant capacity in the lung. Oxidant exposures, like CS can penetrate the epithelial lining fluid, an important barrier to protect the lungs from the environment and direct injure underlying epithelial cells (EpiCs). Interestingly, once the disease is initiated, progression persists even after the cessation of CS exposure, implicating the perpetuation of ROS and free radical formation in the absence of CS. Although the reasons by the pathogenesis continue in spite of the removal of the environmental insult remain unclear, some evidence suggests that particles in the lung are not cleared completely and thus continue driving ROS (Louhelainen, Ryttila et al. 2009). CS induces systematic changes by promoting vascular dysfunction. CS also activates signaling pathways and kinases, leading to the release of mediators ultimately, driving inflammation (Rahman 2005). In addition to directly measuring ROS and their effects on biological molecules, quantifying antioxidant capacity and activity provide important information on the lung's ability to neutralize the effects of ROS.

The lung is equipped with antioxidant machinery that can antagonize the effects

of oxidants like those found in CS. However, disease occurs when this oxidant/antioxidant homeostasis is imbalanced, which can cause significant cellular damage, activation of immune cells and promotion of inflammation (reviewed in (Lee and Thomas 2009)). The lung contains both enzymatic and non-enzymatic antioxidant that neutralize the effects of free radicals. Oxidants activate and stabilize, nuclear factor erythroid 2–related factor 2 (Nrf2), which then translocates to the nucleus where it can activate genes encoding antioxidants. One gene that is upregulated by Nrf2, is reduced glutathione (GSH), which is a non-enzymatic antioxidant that becomes oxidized to its dimeric form, thereby dampening free radicals (Smith, Houston et al. 1993). There is evidence that CS can deplete both the concentration and the activity of antioxidants. Not only do patients with COPD have altered levels of antioxidants (Tavilani, Nadi et al. 2012), but some individuals have genetic polymorphisms encode for suboptimal protein function and are associated with reduced lung function. Splice variants of the enzyme SOD3 have been associated with decreased FEV₁ and increased susceptibility to oxidant-mediated lung injury (Bowler, Nicks et al. 2004, Reinhard, Meyer et al. 2005).

1.6 Cellular Apoptosis & Emphysema Pathogenesis

The “microvascular hypothesis” of COPD has been gaining attention as a mechanism of disease pathogenesis. The pulmonary microvasculature is necessary in maintaining the integrity of alveolar septa (Liebow 1959). This hypothesis states that progressive loss of alveolar structure is caused by direct insults to lung microvasculature, which drives emphysematous tissue remodeling (Barnes 2009, Mackay, Dodd et al. 2013). Histological observations in the emphysematous lung showed significantly

decreased pulmonary capillaries of the alveolar septa and thin alveolar walls, which led to the development of this hypothesis (Liebow 1959). It serves as an alternative to the classical inflammation, protease-antiprotease imbalance hypothesis (Shapiro, 2000), which fails to fully address all of the causes of progressive lung tissue loss in emphysema. More recent clinical, translational and preclinical data have all provided evidence for the loss of pulmonary vasculature in emphysematous destruction. Further, enhanced apoptosis is an established cause of the disappearance of the alveolar septa (Tuder, Zhen et al. 2003).

1.6.1 The Role of Apoptosis in Emphysematous Remodeling

There is a growing body of evidence that demonstrates apoptosis as an important contributor in emphysematous destruction (Schmidt and Tuder 2010). By definition apoptosis is a highly regulated process that describes the collapse of cell due to activation of the of an intracellular death program. Morphologically, it is characterized by cell shrinkage, cytoskeletal collapse, membrane blebbing and DNA fragmentation (Kerr, Wyllie et al. 1972). Both EC and EpiC apoptosis are enhanced in emphysematous remodeling (Kasahara, Tuder et al. 2001). In addition to apoptosis of structural cells that are importance in the maintenance of the integrity of the alveolar septa, immune cells have also been demonstrated to undergo cell death (Hodge, Hodge et al. 2005). Apoptosis initiates damage in a number of ways. EC and EpiC cell death contributes to compromised alveolar structure and function. Concurrent impairment of efficient phagocytosis by neutrophils and monocytes leads to release of pro-inflammatory mediators from apoptotic cells. Inefficient mechanisms of cell clearance also prevent

normal cell replacement (Vandivier, Henson et al. 2006).

CS can initiate cell apoptosis through its capacity to induce double-stranded breaks (DSBs). Increased DSBs lead to the activation of the kinase, ataxia telangiectasia mutated (ATM). ATM directly phosphorylates the histone 2S (H2AX) (γH2AX), ultimately leading to the recruitment and activation of pro-apoptotic downstream effector molecules such as p53 (Canman, Lim et al. 1998, Huang, Halicka et al. 2004). The presence of the DSBs, as indicated by γH2AX foci in alveolar EpiC and ECs was enhanced in the lung of COPD patients as compared to the non-COPD smokers and non-smokers. Amplified DSBs was also associated with increased apoptosis in these lungs (Aoshiba, Zhou et al. 2012). Increased levels of caspase-cleaved cytokeratin-18, a product of apoptotic process have also been correlated with COPD progression (Hacker, Lambers et al. 2009).

The importance of EC in the maintenance of lung homeostasis has been widely demonstrated in the context of COPD, as ECs play a critical role in not only regulating vascular function, but also in maintaining the integrity of the alveolar septa. The microvasculature, which is composed mainly of ECs is responsible for maintain proper alveoli function and is indispensable for EpiC survival (Kasahara, Tuder et al. 2001). There are different models of emphysema that demonstrate the importance of EC homeostasis in the context of emphysematous remodeling.

EC apoptosis in the pathogenesis of emphysema was demonstrated by Giordano and colleagues. They selectively targeted capillary ECs for apoptosis via a peptide-mediated method and induced emphysematous-like tissue destruction in the murine lung (Giordano, Lahdenranta et al. 2008). Another study showed that induced EC apoptosis by

blocking the vascular endothelial growth factor (VEGF) receptor 2 with the pharmacological inhibitor, SU5416 promoted alveolar cell apoptosis and emphysematous tissue damage. Furthermore, inhibiting apoptosis with a caspase inhibitor, antagonized emphysematous tissue destruction, implicating that apoptosis is the specific mechanism that is contributing to remodeling (Kasahara, Tudor et al. 2000). VEGF is critical in EC survival, as it regulates the Akt pathway and downstream anti-apoptotic proteins (Gerber, McMurtrey et al. 1998). It is highly expressed in the lungs and has been differentially expressed in patients with COPD. They demonstrated diminished expression of this angiogenic molecule (Marwick, Stevenson et al. 2006). Additionally, CS exposure suppresses VEGF expression in murine lungs (Wright, Tai et al. 2002). Expression of endothelial nitrous oxide synthase (eNOS) is another marker of has been identified to be diminished in COPD patients (Nana-Sinkam, Lee et al. 2007). Circulating EC progenitor cells (CD34⁺ cells), which are crucial in maintaining lung repair mechanisms, show a 3-fold decrease in COPD patients as compared to their healthy counterparts. Although it is still unclear if it is a causal phenomenon or the result of disease, it provides evidence for altered vascular homeostasis and maintenance in the context of disease (Palange, Testa et al. 2006). Toru and Kubo have also noted the role of circulating microparticles as markers for disease pathogenesis. Microparticles are the result of membrane budding released when different cell types including red blood cells, leukocytes and ECs undergo apoptosis in response to inflammation or injury (Rubin, Canellini et al. 2012). Patients with COPD displayed significantly elevated endothelial cell MP (EMPs) levels as compared to former smokers (Takahashi, Kobayashi et al. 2012). Another larger study stratified patients with COPD according to the GOLD stage progression and has found

that certain types of EMPs correlate with disease severity, implicating EC apoptosis as a marker for disease (Thomashow, Shimbo et al. 2013). Ultimately, these provide evidence for ECs as having an indispensable role in maintaining lung homeostasis. Furthermore, inducing EC apoptosis is sufficient to damage the lung parenchyma, ultimately propelling emphysematous remodeling.

2. Macrophage Migration Inhibitory Factor (MIF)

Macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine that is involved in many different cellular functions. In humans, it is encoded by a single *Mif* gene, located on Chromosome 22. It was initially discovered as a lymphocytic cytokine that inhibited macrophages, but growing literature has demonstrated that MIF is expressed and secreted by most cell types in response to stress (Bernhagen, Calandra et al. 1993). The literature surrounding this cytokine has evolved to elucidate its function as an unconventional cytokine as it has hormonal activities and enzymatic activities in addition to its proinflammatory capacity.

2.1 Structure & Function

MIF is 12.5-kDa protein that consists of 115 amino acid. It was one of the first cytokines discovered in a delayed-type hypersensitivity study as an inhibitor of macrophages secreted from T lymphocyte (Bloom and Bennett 1966). Its functions were made clearer in the 1990's when it was identified as an immune-regulatory cytokine that was secreted and expressed by many different cell types. It was found to inhibit the immunosuppressive effects of glucocorticoids, released in response to many different types of stimuli including lipopolysaccharides, endotoxins and malaria parasite (Donnelly and Bucala 1997, Dobson, Augustijn et al. 2009). MIF can exist as a monomer, homodimer or as a homotrimer. Its homotrimeric structure consists of six α -helices surrounded by three β -sheets forming a barrel-shaped protein. Interestingly, MIF is one

of few proteins that contain a channel that spans the length of the entire protein. The channel has a region of positive electrostatic potential, potentially facilitating its interaction with negatively charged proteins or DNA (Sun, Bernhagen et al. 1996). While MIF exists as a homotrimer, under normal solution conditions, it has the potential to exist as a monomer and homodimer depending on the solvent and concentration (Philo, Yang et al. 2004). Monomer MIF contains two α -helices and six β -sheets (Sun, Bernhagen et al. 1996). MIF binds to its receptor CD74 in its trimeric form (Leng, Metz et al. 2003, Fan, Rajasekaran et al. 2013). Furthermore, Reidy et al showed that the oligomeric state of MIF drives its function and ability to induce inflammatory responses. They showed that trimeric MIF is functionally active and mediates host defense inflammation in response to bacterial infection (Reidy, Rittenberg et al. 2013).

2.1.1 MIF's Cysteine Residues Confer its Oxidoreductase/Enzymatic Capacity

MIF contains three critical cysteine residues (Cys₅₇, Cys₆₀ and Cys₈₀) that exist as free thiol groups (Sun, Bernhagen et al. 1996). In a cross-linkage experiment completed by Mischke colleagues, they found that Cys₆₀ is necessary for MIF's oligomerization, as its substitution with Ser significantly decreased the proportion of homodimers and homotrimers (Mischke, Kleemann et al. 1998). Additionally, these cysteine residues confer MIF's oxidoreductase activity. Mutational studies have revealed that Cys₆₀ being essential for MIF's oxidoreductase activity, while Cys₅₇ is partially responsible for mediating its proper function (Kleemann, Kapurniotu et al. 1999). MIF contains the conserved Cys₅₇-Ala-Leu-Cys₆₀ (CALC or CXXC) motif that is found in other thiol-oxidoreductase proteins such as thioredoxin (Trx). It has been shown the MIF's CALC

motif can catalyze the reduction of small molecules such as insulin and 2-hydroxyethyl disulfide. Furthermore, these two residues play a critical role in regulating MIF's macrophage-activating capacity (Kleemann, Kapurniotu et al. 1998). There is some evidence to suggest that this motif can also regulate intramolecular disulfide bond formation (Sun, Bernhagen et al. 1996). The role of MIF in mediating oxidant injuries has been elucidated in the context of ischemic reperfusion models. MIF^{-/-} mice have increased susceptibility to I/R-induced cardiac injury, which is mediated by an increase in oxidative stress burden, implicating MIF's role in mediating blunting the effects of ROS (Koga, Kenessey et al. 2011). In addition to mediating oxidation-reduction reactions, there is some evidence to suggest that MIF's CALC motif is important in mediating other physiological functions, such as regulating MIF's glucocorticoid inhibitory capacity, potentially through modulation of protein-receptor interaction (Nguyen, Beck et al. 2003). It may however, also impact p53 activity by directly and indirectly regulating the redox status of the cell and reducing intercellular oxidative stress (Nguyen, Lue et al. 2003).

MIF does not behave like a conventional cytokine, in that its enzymatic capabilities are functionally homologous to those found in TPOR superfamily of proteins. In addition to its oxidoreductase ability, it also has tautoisomerase activity. Melanin biosynthesis requires the conversion of the non-physiological compound 2-carboxy-2,3-dihydroindole-5,6-quinone (dopachrome) into 5,6-dihydroxyindole-2-carboxylic acid (DHICA), which was discovered to be catalyzed by MIF (Rosengren, Bucala et al. 1996). It also catalyzes the tautomerization of phenylpyruvate, which is also believed to be biologically insignificant because of its intracellular location and concentration

(Rosengren, Aman et al. 1997). Mutational studies have revealed that Pro₁ is necessary for this activity (Swope, Sun et al. 1998). While targeting MIF's enzymatic capacity may serve as an attractive therapeutic target, the physiological significance of this enzymatic activity is still unclear.

2.1.2 Post-translational Modification & Activity

MIF's third cysteine residue, Cys₈₁ is important in mediating MIF's post-translational modification and its interaction (Luedike, Hendgen-Cotta et al. 2012) with the tumor suppressing transcription factor, p53. Cys₈₁ is necessary for MIF's interaction with p53, mutation of this residue to serine interrupts MIF's binding ability to p53 (Jung, Seong et al. 2008). Cys₈₁ also has the capacity to regulate MIF's oxidoreductase activity, indirectly. This residue can be post-translationally modified by the addition of an s-nitrosylation group (S-NO). MIF^{-/-} mice display enhanced ischemic/reperfusion (I/R)-induced injury in the heart. The treatment of these mice with recombinant MIF (rMIF) attenuated the effects of IR-induced injury. The cardioprotection is lost in Cys81Ser-MIF mutant-treated mice, demonstrating the importance of S-NO-mediated cardiac protection in the context of I/R (Luedike, Hendgen-Cotta et al. 2012).

2.1.3 Secretion & Expression

MIF is constitutively expressed by many different cell types. It is an important mediator of inflammation and is thus expressed by different types of immune cells including monocytes, macrophages, mast cells and dendritic cells. It is also expressed by structural cells such as, EC, EpiCs and fibroblasts (reviewed in (Calandra, Bernhagen et al. 1994, Calandra and Roger 2003)). It has been shown to bind to the CD74 receptor,

activating ERK signaling pathways ultimately, overriding the anti-inflammatory functions of glucocorticoids (Mitchell, Metz et al. 1999, Leng, Metz et al. 2003). MIF may also mediate its biological effects via endocytosis, a receptor-independent mechanism (Kleemann, Hausser et al. 2000). Given MIF's critical role in modulating immune responses it is ubiquitously expressed throughout most organs tissues of the body, but highly expressed in tissues that interface with the environment, such as the lungs and gut and expressed in organs that are important regulating immunity, including the spleen, lymph nodes, thymus and bone (The Human Protein Atlas, accessed June 2017).

MIF's role as a potent regulator of inflammation and immune responses to varying microbial toxins, infections, other proinflammatory cytokines and glucocorticoids has been well-established (Calandra, Bernhagen et al. 1995, Bacher, Meinhardt et al. 1997). It is rapidly released in the setting of infections as it regulates innate, acquired and autoimmune responses. Upon activation and secretion, MIF exhibits both paracrine and autocrine functions in the context of inflammation. It goes into circulation where it leads to the expression of the pro-inflammatory cytokines tumor necrosis factor- α (TNF- α , IFN- γ , IL-1 β and IL-6). It can activate macrophages and T cells and inhibit apoptosis (Lue, Kleemann et al. 2002, Bucala and Donnelly 2007).

2.2 MIF, Apoptosis & Emphysematous Remodeling

MIF's role in inhibiting apoptosis has gained attention in the context of emphysema/COPD. Circulating plasma levels of MIF are decreased in smokers with COPD as compared to smokers without COPD (Sauler et al. 2013). Furthermore, this phenomenon is inversely correlated with disease severity as patients with advanced stage COPD (GOLD Stage V) had the lowest levels of circulating MIF (Fallica et al. 2014). While it is unclear if dampened plasma MIF level is a causal factor of disease or merely an epiphenomenon, preclinical data suggests a causal relationship. Preclinical data has further elucidated MIF's role in emphysema pathobiology. MIF is differentially expressed in the context of CS exposure. Under conditions of acute exposure, MIF expression is upregulated in the BAL and murine lung (Sauler et al, 2013). Chronic CS exposure, however suppresses MIF expression, which is coincident with emphysematous tissue destruction *in vivo* (Fallica et al, 2014, Sauler et al, 2013). The knockout of MIF *in vivo* also further sensitizes mice to the effects CS. These mice have enhanced airway remodeling, characterized by greater air space enlargement (Fallica Boyer et al, 2014, Sauler et al, 2013). Further, these animals have increased expression of γ H2AX, ROS production and CS-induced apoptosis, demonstrating MIF's role in antagonizing these events (Fallica, Boyer et al. 2014; Fallica Varela et al, 2016).

While both EpiCs and ECs are susceptible to CS-induced apoptosis, Fallica et al found that MIF deficiency preferentially enhances CS-induced apoptosis in ECs (Fallica et al, 2014). EC apoptosis has been determined to be both a necessary and sufficient process that drives the progression of emphysematous tissue remodeling and COPD,

which is characterized by increased cell apoptosis that outpaces repair (Liu, Ding et al. 2014, Strulovici-Barel, Staudt et al. 2016). MIF's role in regulating EC homeostasis has been previously demonstrated in other studies. Munat and colleagues showed that MIF and VEGF levels are strongly correlated in the context of glioblastoma cells (Munaut et al. 2002). The effects of MIF on EC apoptosis were first identified by Damico et al. They showed that the loss of MIF results in the loss of EC resistance to lipopolysaccharide (LPS)-induced apoptosis, implicating its role as a necessary regulator of EC survival. They also showed that MIF regulates its cytoprotection through FLICE-like inhibitor protein (FLIP), an antagonist of the death receptor-mediated apoptosis (Damico, Chesley et al. 2008). MIF's ability to antagonize ECs apoptosis was also demonstrated in the context of CS *in vitro*, via inhibition of p53 activation (Damico et al. 2010). p53 is a tumor suppressor that induces cell cycle arrest and apoptosis in response to genotoxic stimuli (Nigro, Baker et al. 1989). CS exposure induces EC apoptosis in a p53-dependent manner. This was evidenced by the siRNA-mediated knockdown of p53 *in vitro*, which conferred apoptotic resistance in ECs exposed to CS. Furthermore, MIF deficiency in these cells led to p53 upregulation and exacerbated sensitivity of EC's to CS. Rescue experiments that demonstrated the pharmacological inhibition of p53, demonstrated MIF's ability to antagonize CS-induced is p53-dependent (Damico, Simms et al. 2011). Additionally, MIF's role in antagonizing p53-mediated EC apoptosis is through its ability to directly inhibit activity of apoptosis signal kinase 1 (ASK1) and downstream activation of the p38 mitogen activated kinase (MAPK) pathway (Fallica, Varela et al. 2016).

Activity of the ROS-generating enzyme, xanthine oxidoreductase (XOR) is elevated in lungs of mice exposed to CS (Kim et al. 2013). ASK1 drives XOR activity

and MIF has been shown to antagonize this. MIF^{-/-} mice have exacerbated XOR during CS exposure. The increased activity of XOR is associated with increased ROS production in the murine lung. Furthermore, *in vitro* data reveals that MIF antagonizes ASK1-dependent phosphorylation of p38, subsequent XOR activation, increased ROS accumulation and DNA damage-induced p53 activation (Fallica, Boyer et al. 2014). In conclusion these provide evidence for antagonizing the effects of CS-induced emphysematous remodeling via its ability to maintain EC homeostasis and confer apoptotic resistance in the context of ROS.

2.3 The Emerging Role of MIF as a Positive Regulator of Antioxidant Responses

MIF's complex role as an antagonist of apoptosis has been partially elucidated by a growing body of literature that demonstrated its ability to regulate antioxidant responses. It has been established that MIF's oxidoreductase activity can directly regulate cellular redox status. It has been shown to confer cytoprotection through its ability to mitigate the effects increased oxidative burden, ultimately antagonizing DNA damage and p53 activation. MIF protects HeLa cells from the effects of oxidant-induced apoptosis via its Cys₆₀ residue. Further, it positively regulates antioxidants and induces intercellular levels of glutathione and glutathione S-transferase (Takahashi, Nishihira et al. 2001, Nguyen, Beck et al. 2003).

Upregulation of antioxidants is crucial in promoting cytoprotection in the context of oxidative stress. Nrf2, a critical regulator of the antioxidant response element (ARE), is critical for the regulation of a variety of antioxidant genes (Moi, Chan et al. 1994). MIF's ability to regulate the ARE was first identified by Kimura and colleagues. They

showed that the novel cardioprotective drug 1,3-benzothiazin-4-one (BTZO-1) inhibits cardiac myocyte apoptosis in the context of heart disease via direct binding to MIF (Kimura, Sato et al. 2010). BTZO-1 promotes cell survival by upregulating ARE-dependent gene expression. Treatment of cardiomyocytes with exogenous MIF inhibited nitric oxide-induced apoptosis. Furthermore co-stimulation of the drug and exogenous MIF, increased ARE activity and upregulated the ARE-dependent transcript, hemoxygenase-1 (HO-1), implicating a MIF-dependent mechanism for optimal cytoprotective function of BTZO-1 (Kimura, Sato et al. 2010). In addition to modulating ARE activity, MIF has been shown to impact Nrf2 expression. MIF deficiency potentiates susceptibility to radiation-induced lung injury (RILI) in an age-related manner. MIF deficient mice had worse lung injury, attenuated antioxidant capacity as well as, Nrf2 protein expression in the lung. Treatment of MIF^{-/-} mice with antioxidants protected them from RILI, implicating that enhanced susceptibility in these is due to altered Nrf2-antioxidant responses. MIF loss *in vitro* decreased nuclear translocation of Nrf2 in ECs challenges with hydrogen peroxide (H₂O₂). These data provide evidence for MIF's function upstream of the ARE and highlight MIF's alternative cytoprotective role in inhibiting apoptosis and mitigating oxidative responses via induction of antioxidant-dependent mechanisms.

3. Oxidants and Antioxidant Responses

3.1 Oxidative Stress, Antioxidants

Oxidative stress is a state in which the effects of oxidants overwhelm the antioxidant capacity in a given cellular milieu or organ system. Oxygen is integral to aerobic energy production, which is far more efficient than anaerobic respiration. However the use of oxygen during aerobic respiration can lead to the production of chemically reactive compounds known as reactive oxygen species (ROS) (reviewed from ((Bowler, Barnes et al. 2004)). Free radicals, which include ROS and can perpetuate oxidative stress, are defined as an atom or molecule with an unpaired electron (Cheeseman 1993).

ROS and other types of free radicals are highly unstable and reactive and can attack most organic biological macromolecules if kept unchecked by a cell's antioxidant defense system. Gutteridge defined an antioxidant:

“any substance that, when present in low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits the oxidation of that substrate” (Gutteridge 1995).

He further subdivided antioxidants in three broad categories based on their functions: antioxidant enzymes, chain breaking antioxidants and transition metal binding proteins (Halliwell and Gutteridge, 2006). Antioxidant enzymes can either donate an electron or receive an electron, becoming oxidized or reduced, respectively (Halliwell, 1995). Chain breaking antioxidants are non-enzymatic antioxidants that are particularly important in the prevention of chemical chains that form as a result of a reactive molecule

interacting with an organic macromolecule (i.e. lipid peroxidation). Finally, transition-binding proteins sequester metals such as copper or iron to inhibit the formation of radicals (reviewed in (Young and Woodside 2001, Bowler, Barnes et al. 2004)).

The imbalance between oxidants and antioxidants diseases leads to oxidative stress and is the underlying cause of many diseases, including pulmonary and airway diseases. The lungs are particularly susceptible to the effects of ROS due to their large surface area and incessant exposure to both endogenous and endogenous sources of oxidants and pro-oxidants in approximately 8,000 liters of air, daily. There are established acute and long-term effects on pulmonary function in response to environmental sources of ROS (Bernstein, Alexis et al. 2004). Acute exposure to oxidants can cause reversible decrements in lung function (Hollingsworth, Maruoka et al. 2007), but chronic exposure can lead to irreversible remodeling and decreased pulmonary function (Bernstein, Alexis et al. 2004). There is mounting evidence that show oxidative stress is involved in the pathogenesis of many chronic pulmonary conditions. Markers of oxidative stress are significantly elevated in patients with chronic conditions like COPD (Calikoglu, Unlu et al. 2002, Corradi, Rubinstein et al. 2003). Furthermore, oxidants have the capacity to enhance inflammation, promoting disease and pulmonary dysfunction therefore addressing the oxidant/antioxidant imbalance is critical in different therapeutic approaches (MacNee 2001, Hoshino and Mishima 2008).

3.1.1 Sources of ROS and their Effects on Cellular Function

ROS is vital to cellular function, but its excess can quickly induce damage. While it is essential for the maintenance of normal physiological functions, overexposure to

ROS can promote disease and lead to detrimental health outcomes. ROS can be broadly divided into two basic categories, endogenous and exogenous sources of ROS. Endogenous sources of ROS arise from organelles or processes that occur within the cell. The mitochondria is the site of aerobic respiration and a potent intracellular ROS-generating source (Loschen, Flohe et al. 1971, Boveris, Oshino et al. 1972). In moderate concentrations, free radicals are important in cell-to-cell communication via perpetuation of signal transduction pathways (Thannickal and Fanburg 2000). ROS and free radicals are also employed by the immune system in host defense mechanisms and cytokine activation (Lo, Wong et al. 1996, Droge 2002).

Exogenous sources of ROS, pro-oxidants reactive nitrogen species include ambient air pollution, occupational exposures (industrial solvents), biomass burning and CS exposure (reviewed in (Sies, Berndt et al. 2017). Excessive exposure to can quickly tip the homeostatic balance and lead to oxidative stress. Under these conditions, free radicals can rapidly react with cellular molecules. A consequence of oxidative stress is lipid peroxidation, which is caused by a chain reaction of poly-unsaturated lipids ultimately, leading to permanent cell membrane damage and cell death (Chen, Arjomandi et al. 2007). DNA damage is another consequence of excess ROS exposure and plays an central role in many pathologies (Jena 2012). Free radicals can be carcinogenic as they can cause DNA strand breaks, backbone lesions and base modifications, potentially inducing DNA mutations, transformation and potentiation of cancer (Balasubramanian, Pogozelski et al. 1998). Finally, ROS can attack protein and disrupting or inhibiting their function. Oxidation of proteins can lead to protein-protein cross-linkages, proteolytic degradation and effect protein turnover (Davies 1986). In conclusion, excess ROS

damages intracellular macromolecules, induces cellular dysfunction ultimately leading to inflammation and disease.

3.2 Antagonism of ROS Effects by the Antioxidant Response Element (ARE)

Antioxidant responses are due the upregulation of cytoprotective genes during oxidative stress due to the activation of the antioxidant response element (ARE) (Tsuji, Ayaki et al. 2000). The ARE are cis-acting enhancer sequences found in the promoter regions of many antioxidant genes. These genes encode for proteins that protect the cell from oxidative stress (Li and Jaiswal 1992, Nguyen, Sherratt et al. 2003). The ARE was first identified in rats, originally termed β - naphthoflavone- responsive element between. This inducible response element is located between nucleotides -722 and -682 in the 5'-flanking region of the rat glutathione S-transferase Ya subunit gene (Rushmore, King et al. 1990). Rushmore and Pickett found that it is distinguishable from other xenobiotic response elements by its responsiveness to phenolic antioxidants; hence it was termed antioxidant response element or ARE (Rushmore and Pickett 1990). They have shown that this element can be induced by P-naphthoflavone and *t*-butylhydroquinone, thereby, activating Ya subunit gene. It is activated by a number of different compounds that share common characteristics. These compound or chemicals can undergo redox cycling, or they can be enzymatically converted to more reactive intermediate (Nguyen, Nioi et al. 2009).

4. Nuclear factor (erythroid-derived)–like 2 (Nrf2)

4.1 Discovery of Nrf2

Nrf2 is a member of the cap'n'collar (CnC) subfamily of proteins of the family. CnCs belong to a superfamily of proteins known as the basic-region leucine zipper (bZIP) (Mohler, Vani et al. 1991). CnC family of proteins consists transcription factors that are conserved among different species. It consists of BACH 1 and 2 as well as, NF-E2 p45, Nrf1, Nrf2, and Nrf3 (Oyake, Itoh et al. 1996). They have a characteristic 43-amino acid CnC domain (Bowerman, Eaton et al. 1992). These transcription factors play important roles in development, as well as stress responses including oxidative stress (Chan, Cheung et al. 1995). Cells are thus equipped with mechanisms to both sense (Cullinan, Gordan et al. 2004) and manage oxidative stress. The CnC member, Nrf2 serves as critical transcription factor in activating a host of antioxidants in response to oxidant-induced stress.

Initially, Nrf2 was discovered as a binding partner of activating protein (AP)-1 and nuclear factor-erythroid 2 (NF-E2) (Moi, Chan et al. 1994). The function of Nrf2 was largely unknown, as the absence of the Nrf2 gene in a murine model did not have any development effects, nor did it disrupt physiology (Chan, Lu et al. 1996). The function of Nrf2 was later elucidated by Ito and colleagues in a study that investigated the effects of a former food additive, butylated hydroxyanisole (BHA) on the induction of detoxifying enzymes, GST and NQO-1 in Nrf2-deficient mice. It was found that the absence of Nrf2 greatly diminished expression of both GST and NQO-1, demonstrating its role in upregulating antioxidant gene transcription (Itoh, Chiba et al. 1997).

4.2 Nrf2 Structure: The Seven Domains of Nrf2

Nrf2 is a member of the bZIP family of proteins and as such contains two important motifs critical for its function: a DNA binding motif and a positively charged leucine zipper. The leucine zipper interacts with the major grooves of DNA at the N-terminal (Alber 1992, Ellenberger 1994). The bZIP region is characterized by a thirty amino acid sequence that is rich in the basic residues and a the leucine zipper, which 30-40 amino acids in length (Vinson, Sigler et al. 1989). Human Nrf2 (hNrf2) is comprised of 605 amino acids, which form seven domains known as the Nrf2_ECH homology (Neh) domains (Ellenberger 1994). The Neh1 domain contains the CNC-b-ZIP region at the C-terminal half, which contains the leucine zipper motif and is crucial for binding to the ARE (Itoh, Wakabayashi et al. 1999). In addition to its role in DNA binding, the CnC domain of Nrf2 interacts with bZIP motifs of member of the small masculoaponeurotic fibrosarcoma (Maf) family of proteins (Motohashi, Katsuoka et al. 2004). Heterodimerization with these proteins facilitates Nrf2 binding to the ARE and are essential for optimal induction of ARE-dependent genes (Katsuoka, Motohashi et al. 2005).

Domains Neh2 and Neh6 are important in the negative regulation of Nrf2. Neh2 interacts with the repressor of Nrf2, Keap1 via two highly conserved motifs, DLG and ETGE (Tong, Katoh et al. 2006, Chowdhry, Zhang et al. 2013). KEAP1 forms a homodimer with Nrf2, with each monomer binding with low affinity to DLG high affinity to ETGE (Tong, Kobayashi et al. 2006). The DLG (or DIDLID) element spans amino acids 17-32 and is necessary for KEAP1-induced degradation of Nrf2. The ETEG

domain (amino acids 79-82) facilitates KEAP1-Nrf2 interaction under basal homeostatic conditions (McMahon, Thomas et al. 2004). The addition of a poly-ubiquitin chain to Nrf2 occurs at lysine residues within the Neh2 domain is about 10-30 amino acids away from the N-terminal of ETGE motif (Zhang, Lo et al. 2004). Phosphorylation of the threonine residue 80 of the ETGE domain is sufficient to perturb the interaction between KEAP1 and Nrf2 by impeding the ETGE motif from appropriately fitting into the binding pocket of KEAP1 (Kong, Owuor et al. 2001, Lo, Li et al. 2006).

Unlike Neh2, Neh6 negatively regulates Nrf2 independent of KEAP1. Neh6 contains the DSGIS motif, which is a redox-insensitive degron and ensures rapid Nrf2 turnover under conditions of oxidative stress (McMahon, Thomas et al. 2004). It is converted to a phosphodegron by the enzymes glycogen synthase kinase-3 (GSK-3), which targets it for phosphorylation and subsequent activation (Salazar, Rojo et al. 2006, Rada, Rojo et al. 2011). This then allows for the recruitment of the protein β -transduction repeat containing protein (β -TrCP) via the Neh6 domain motifs, DSGIS (338) and DSAPGS (378) (Salazar, Rojo et al. 2006). The interaction between Nrf2 and β -TrCP is significantly enhanced when DSGIS is phosphorylated (Chowdhry, Zhang et al. 2013). The adaptor molecule S-phase kinase-associated protein 1 (Skp1) adaptor protein interacts with the F-box domain of β -TrCP. This adaptor molecule connects Neh6 of Nrf2 to the Cull1-Rbx1 core E3 complex, providing another mechanism for Nrf2 ubiquitination. Neh6 has been demonstrated to destabilize Nrf2 in redox-independent manner, thereby enhancing Nrf2 degradation under conditions of oxidative stress (McMahon, Thomas et al. 2004, Rada, Rojo et al. 2011). Chowdhry et al. found that deleting Neh2 and two motifs of the Neh6, SDS1 and PEST, significantly increased Nrf2

activity (Chowdhry, Zhang et al. 2013). Another domain involved in the negative regulation of Nrf2 is the Neh7 domain. Amino acids 209–316 of human Nrf2 comprise Neh7 and it is the site of interaction with retinoic X receptor alpha (RXR α), a repressor of the protein. The knockdown of RXR via siRNA enhanced ARE-dependent gene transcription under basal conditions and stimulation with tert-Butylhydroquinone (TBHQ) (Wang, Liu et al. 2013).

Neh3 is located at the C-terminal domain half of the protein and has been shown to play an important role in regulating transcriptional activation of Nrf2. The Neh3 domain allows for proper binding to chromodomain helicase DNA-binding protein 6 (CHD6), via its VFLVPK motif. Mutation of this hydrophobic motif not only reduces protein-protein interaction with CHD6 but also the decrease Nrf2 activity, conferring its importance in the transcriptional activation of Nrf2. Also, the deletion of the final 16 amino acids of Nrf2 renders the protein transcriptionally inactive, although it may still localize in the nucleus and bind to DNA (Nioi, Nguyen et al. 2005). Neh4 and Neh5 are important for optimal transcriptional activation of Nrf2. Katoh et al. demonstrated that deletion of Neh4 and Neh5 significantly abrogated Nrf2-dependent ARE-activity (Katoh, Itoh et al. 2001) and deletion of latter lead to a significant attenuation of Nrf2-dependent transcription of *HO-1* (heme oxygenase 1), *NQO1* (NAD(P) H:quinone oxidoreductase 1) and *GCLM* (glutamate cysteine ligase modulatory subunit) (Zhang, Hosoya et al. 2007). Furthermore, these two domains can bind independently and cooperatively to CREB (cAMP Responsive Element Binding protein) Binding Protein (CBP) (Katoh, Itoh et al. 2001). Not only does CBP functions act as a co-activator of many other molecules, but it also has an intrinsic histone acetylase activity important in chromatin remodeling and

enhancement of gene transcription (Bannister and Kouzarides 1996, Goodman and Smolik 2000). Inhibition of CBP markedly reduces ARE-activity. Additionally, Wang et al. demonstrated that overexpressing RXR may disrupt the binding of CBP to Neh4 and Neh5 and subsequently decreased transcription by Nrf2 (Wang, Liu et al. 2013). In addition to facilitating the recruitment of CBP, Neh5 has been shown to interact with Brahma-related gene-1 (BRG), which plays an important role in ATP-dependent chromatin remodeling (Ramirez-Carrozzi, Nazarian et al. 2006). Under conditions of oxidative stress, Nrf-2 recruits BRG1 and subsequently, RNA polymerase II to the promoter of HO-1 (Zhang, Ohta et al. 2006), which is enhanced by the Neh5 domain (Zhang, Hosoya et al. 2007). In summary, Nrf2's domains function to both positively and negatively regulate Nrf2 protein to ensure its efficient turnover under varying cellular redox conditions.

4.3 Regulation of Nrf2

4.3.1 Transcriptional Regulation

There are several ways in which Nrf2 is regulated at the transcriptional level. Nrf2 is encoded by the *NFE2L2* gene and is regulated a number of different ways (Moi, Chan et al. 1994). It has been determined that the aryl hydrocarbon receptor (AHR)-xenobiotic response element signaling pathway directly affects *Nrf2* gene expression (Radjendirane and Jaiswal 1999, Miao, Hu et al. 2005). The AHR is a ligand-activated transcription factor that is responsible for activating phase I and II genes in the presence of planar aromatic hydrocarbons. AHR forms a complex with aryl hydrocarbon receptor nuclear

translocator (Arnt) and binds to the promoter of the xenobiotic response element (XRE) (Rushmore and Kong 2002). It was initially believed that the ARE and XRE pathways are distinct, Jaiswal et al showed that there is in fact crosstalk and regulation between the two. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a known inducer of the XRE pathway was also shown to induce the ARE-dependent gene expression of NQO-1 (Radjendirane and Jaiswal 1999, Ma, Kinneer et al. 2004). Additionally, Miao showed that TCDD directly increases *Nrf2* gene expression and that this effect is absent in AHR-deficient cells. They also studied the effects of TCDD on *Nrf2* gene in CYP1A1-deficient cell line c37 and found that *Nrf2* is upregulated independent of CYP1A1 (Radjendirane and Jaiswal 1999). In addition to AHR regulation of *Nrf2* gene expression, epigenetics plays a role in the suppression of Nrf2. Hypermethylation of specific CpG sites in the Nrf2 gene promoter represses *Nrf2* gene expression and its downstream antioxidant gene targets (Yu, Khor et al. 2010).

4.3.2 Post-Translational Regulation-Degradation of Nrf2

Nrf2 may be post-translationally modified and subsequently regulated in a number of ways. Nrf2 levels are maintained under homeostatic conditions by the adaptor molecule KEAP1. KEAP1 serves as the substrate of Cullin3-E3 ubiquitin ligase complex that ultimately connect the poly-ubiquitin chain to Nrf2, targeting it for degradation. Nrf2 may also be modified by phosphorylation. This thesis will focus on the degradation pathway of Nrf2 by KEAP1.

4.3.2.1 Keap1: The Key Suppressor of Nrf2

Kelch-like ECH-associated protein 1 (KEAP1) is dominant suppressor of Nrf2 protein. In the absence of oxidative stress, Keap1 sequesters Nrf2 in the cytoplasm and targets Nrf2 for proteasomal-mediated ubiquitination (Zhang, Lo et al. 2004). Oxidative stress leads to the disassociation of Nrf2 from and Keap1 and subsequent translocation to the nucleus. The structure of KEAP1 is critical for its function as it has the capacity to sense redox alterations in the cell (Canning, Sorrell et al. 2015). It serves as an adaptor molecule that binds to the scaffold protein, Cullin 3 to form the Cullin-RING E3 ligase (CRL3) complex, which is required for the poly-ubiquitination and degradation of Nrf2 (Cullinan, Gordan et al. 2004).

4.3.2.2 Structure and Function of KEAP1

KEAP1 is a cysteine-rich molecule that is comprised of 625 amino acids (Tkachev, Menshchikova et al. 2011). It consists of five domains: (I) N-terminal (amino acids 1-60) (II) the broad complex, tramtrack and bric-a-brac domain (amino acids 61-179), (III) the intervening region (IVR) (amino acids 180-314), (IV) the Kelch repeat (or double glycine repeats (DGR)) (six Kelch repeats amino acids 315–359, 361–410, 412–457, 459–504, 506–551, and 553–598), (V) and the C-terminal region (CTR) (amino acids 599–624) (Adams, Kelso et al. 2000, Dinkova-Kostova, Holtzclaw et al. 2002). It is a member of the Broad-complex, Tramtrack and Bric-à-brac/poxvirus and zinc finger (BTB/POZ) family of proteins. The evolutionarily conserved BTB motif is about ~120 amino acids in length and is crucial in facilitating protein-protein interactions, forming homo and heterodimers (Bardwell and Treisman 1994), regulating gene transcription

(Stogios, Downs et al. 2005), scaffolding to the cytoskeleton (Kang, Kobayashi et al. 2004) and substrate recognition for proteasomal-mediated degradation (Furukawa, He et al. 2003). In the case of KEAP1, the BTB domain is responsible for the recognition and recruitment of Cullin 3 (CUL3) a member of Cullin–RING (really interesting new gene) family of ligases (Cullinan, Gordan et al. 2004, Hudson and Cooley 2010). It also plays a critical role in KEAP1 homodimerization and a point mutation of its highly conserved Ser104 residue leads to impaired homodimerization and Nrf2-binding (Zipper and Mulcahy 2002).

The BTB/POZ family of proteins is sub-divided into different subclasses and KEAP1 is a member of the BTB-Kelch subclass, which consist of 49 members that are characterized by the presence of the BTB domain at the N-terminal and Kelch domain at the C-terminal (Stogios and Prive 2004). The crystal structure of the Kelch domain in KEAP1 reveals a four-beta anti-parallel sheet arrangement, forming a six-bladed β -propeller. All of six blades that form the β -propeller interact with Nrf2 and while these blades vary in amino acid length, they all contain a conserved 7-residue sequence. The longer, blade II is crucial for the KEAP1-Nrf2 interaction (Li, Zhang et al. 2004). The Kelch domain of KEAP1 binds two Nrf2 molecules, simultaneously via Nrf2's conserved ETGE motif with the Neh2 domain. Phosphorylation of this motif significantly hinders KEAP1 bind capacity to Nrf2. In addition to the DGR domain, the C-terminal has also been shown to be critical in mediating KEAP1-Nrf2 interactions (Padmanabhan, Tong et al. 2006).

KEAP is a cysteine-rich molecule that contains 25 and 27 cysteine residues in murine and human KEAP1, respectively. These residues are crucial in detecting changes

in the redox status of a cell and they facilitate KEAP1-Nrf2 interaction. Furthermore, the cysteine residues are concentrated within the IVR of the molecule, which contains 8 Cys over the span of about 100 amino acids. The most reactive Cys residues identified by Dinkova et al. are Cys₂₅₇, Cys₂₇₃, Cys₂₈₈, and Cys₂₉₇, via exposure to an established inducer of Phase II enzymes, dexamethasone (Dinkova-Kostova, Holtzclaw et al. 2002). Cys₁₅₁, Cys₂₅₇ and Cys₂₈₈ are the residues critical in the interaction between Nrf2 and KEAP1 and are involved in the proteasomal-mediated degradation of Nrf2. Point mutations of these cysteine residues to serine led to the accumulation of Nrf2 in COS2 cells exposed to tBHQ. Cys₁₅₁ however, is responsible for the stabilization and redox-induced release of Nrf2 and mutation of this residue led to the constitutive repression of Nrf2 (Zhang and Hannink 2003). The physiological importance of the Cys₁₅₁, Cys₂₇₃ and Cys₂₈₈ in the IVR was established in KEAP1 null mice that transgenically expressed mutant Keap1 (C273A) and/or Keap1 (C288A) failed to inhibit constitutive Nrf2 activity (Yamamoto, Suzuki et al. 2008). These specific Cys residues are responsible for redox sensing and responding to oxidative stress (Takaya, Suzuki et al. 2012, Saito, Suzuki et al. 2015).

4.3.2.3 Proposed Models of KEAP1 and Nrf2 Release

KEAP1's function is two-fold, first it senses changes in the redox status of the cell and it tightly regulates Nrf2 expression through the facilitation of its ubiquitination. The interaction between these two molecules is perturbed by the presence of electrophiles. There are several proposed models of KEAP1 interaction with Nrf2. The chief two models are KEAP1-cullin3 (CUL3) disruption model (Eggler, Small et al.

2009) and the “two-substrate recognition model” (also known as the “hinge latch model”) (McMahon, Thomas et al. 2006, Tong, Katoh et al. 2006). The latter has become the more widely accepted prevailing model.

The KEAP-CUL3 disruption model proposes that the modification of Cys₁₅₁ of KEAP1's BTB domain causes a conformational change and disrupts the interaction between KEAP1 and CUL3. This disruption then inhibits the poly-ubiquitination and degradation of Nrf2 (Rachakonda, Xiong et al. 2008, Egger, Small et al. 2009). KEAP1 serves as an adaptor molecule that facilitates the transfer of ubiquitin from cullin3 (CUL3)/Rbx1 ligase complex to the lysine residues of the Neh2 domain of Nrf2 (Kobayashi, Kang et al. 2004). Zhang et al demonstrated that KEAP1 forms a ternary complex with the scaffold protein, CUL3 and Rbx1, which is mediated by the BTB domain. Furthermore, mutations of specific residues within the BTB domain reduced KEAP1's interaction with CUL3-Rbx1 complex in a co-immunoprecipitation experiment (Zhang, Lo et al. 2004). It was then shown that Cys₁₅₁ is not only important in sensing redox changes, but also plays a role in regulating KEAP1 and CUL3 interaction. Substitution of Cys₁₅₁ to the bulkier amino acid, tryptophan disrupted the KEAP1's binding with CUL3 and enhanced ARE activity. The tryptophan substitution caused steric clash with residues K131 and R135 between the alpha helix they reside on and the alpha helix that Cys₁₅₁ resides on, ultimately shifting the residues that interact with CUL3 and disruption KEAP1-CUL3 interaction (Egger, Small et al. 2009).

The prevailing more widely accepted model is the “hinge and latch” model. This model demonstrates KEAPs binding to the two motifs of the Nrf2's Neh2 domain, ETGE and DLG (Tong, Katoh et al. 2006). KEAP1 form a homodimer and interacts with these

two binding sites with different affinities. KEAP1 binds to the ETGE motif with about two orders of magnitude higher affinity than the DLG motif (McMahon, Thomas et al. 2006). The “hinge” in this model is the interaction of the Kelch domain of Keap1 with the ETGE motif of Nrf2’s Neh2 domain and the “latch” is the interaction of KEAP1 with the DLG motif. The hinge anchors Keap1 onto Nrf2 and the lesser affinity DLG acts a “latch” by changing the position of Nrf2 depending on the cell’s redox state (Tong, Kobayashi et al. 2006). One KEAP1 molecule of the homodimer binds to each motif separately. McMahon and colleagues showed that deletion of the DLG motif inhibits the ubiquitination of Nrf2, but does not impact KEAP1’s binding via the ETGE motif, implicating its role as a mediator of ubiquitination (McMahon, Thomas et al. 2006).

Under conditions of oxidative stress cysteine residues become oxidized leading to a conformational change, misalignment of the lysine residues (Kansanen, Kuosmanen et al. 2013) and subsequent release the DLG motif “latch.” In this manner, Nrf2 is released from KEAP1 and no longer targeted for proteasomal-mediated degradation. It therefore, accumulates in the cytoplasm or translocates to the nucleus. This model suggests that Nrf2 may still be stabilized while still bound to KEAP1, but not targeted for degradation so that newly translated Nrf2 bypasses KEAP1, altogether. Interestingly, the type of electrophilic inducer may play a role in directly impacting Nrf2-KEAP1 interactions and promote the disassociation of Nrf2 from KEAP1 (Tong, Katoh et al. 2006, Tong, Kobayashi et al. 2006, He, Lin et al. 2007). While the exact mechanism by KEAP1 relinquishes Nrf2 has yet to be determined, mounting evidence suggests that the “hinge-latch” model best explains KEAP1-Nrf2 disruption in the context of oxidative stress.

5. Cullin E3 RING Ligases & their Role in the Polyubiquitination of Nrf2

KEAP1 does not directly poly-ubiquitinate Nrf2, instead it merely serves as an adaptor of the Cullin3-E3 ligase complex that connects ubiquitin chain to Nrf2. Ubiquitination leads to the formation of poly-UB that is recognized by the 26S proteasome (De Mot, Nagy et al. 1998). It begins with the activation of a highly conserved 76-amino acid ubiquitin (Ub) molecule (Komander 2009) molecule by ubiquitin-activating enzyme (E1). Activation entails the thio-ester bond formation between the C-terminal of a Ub molecule and a Cys residue of the E1's active site (Schulman and Harper 2009). This activated Ub molecule is then transferred to a ubiquitinating enzyme (E2) (Olsen and Lima 2013) and finally to protein ligase (E3) (Komander 2009).

There are two main families of E3 ligase, Homologous to E6-AP Carboxy Terminus (HECT) and Really Interesting New Genes (RING), which facilitate ubiquitination via different mechanisms. HECT E3 ligases form an intermediate thioester bond with Ub before ubiquitinating the target substrate (Rotin and Kumar 2009, Metzger, Hristova et al. 2012). RING E3 ligases function differently and serve critical roles in substrate recognition (Lydeard, Schulman et al. 2013). RING family of E3 ligases represents the largest family of E3 ubiquitin ligases. They are responsible for the ubiquitination and clearance of about 20% of proteins (Soucy, Smith et al. 2009). Humans express eight members of the Cullin family: CUL1, CUL2, CUL3, CUL4a, CUL4b, CUL5, CUL7 and CUL9 (Petroski and Deshaies 2005, Cui, Xiong et al. 2016). The basic anatomy of CRLs is: a cullin that serves as the central scaffold, Ring-

finger protein (RING), such as RBX1 or RBX2, an adaptor molecule and a substrate (Cui, Xiong et al. 2016). The diversity of the CRLs is due to the presence of multiple cullin molecules that recognize various protein substrate motifs. The N-terminal domain (NTD) binds to the adaptor molecule, which serves to recognize the substrate and connect it to the CRL. The C-terminal domain (CTD) binds to the RING member protein and is the site of post-translational modification (PTM) via the addition and removal of a Nedd8 molecule (Duda, Borg et al. 2008). This process of cyclic neddylation and deneddylation regulates CRL activity (Kawakami, Chiba et al. 2001).

5.1 The Role of CRL3 Subclass in Nrf2 Regulation

Cullins are critical for organizing the CRL's into functional units, as they serve as the central scaffolds that bind to E3 at C-terminal. CUL3 plays an indispensable role in murine embryogenesis and its knockout confers embryonic lethality (Singer, Gurian-West et al. 1999). CUL3's importance in cell cycle regulation, meiosis (Pintard, Kurz et al. 2003) and mitosis have been demonstrated in *C. elegans* and mammalian cells (Sumara, Quadroni et al. 2007). This complex also plays important roles in cell apoptosis (Lee, Yuan et al. 2010) and transcriptional stress responses through the recognition and binding of the BTB protein, KEAP1 (Eggler, Small et al. 2009).

Cullins are capable of ubiquitinating the target molecule directly or indirectly, facilitated by an adaptor molecule bound to the NTD. In the case of CRL3, CUL3 binds the redox sensor and adaptor molecule, KEAP1 and polyubiquitinates Nrf2 (Kleiger, Saha et al. 2009). CRL3 specifically assembles around the CUL3, which

uniquely recognizes the Kelch β -propeller domain of BTB/POZ motif (Ahmad, Engel et al. 1998, Canning, Cooper et al. 2013). This family of protein contains about 50 members Kelch-like 1–42 (KLHL1–42) and Kelch and BTB domain-containing 1–14 (KBTBD1–14). KEAP1 is a member of this family and is an established CUL3 adaptor molecule (Dhanoa, Cogliati et al. 2013). KEAP1 forms dimer with two CUL3 molecules, thereby forming a dimeric CLR3, which is essential for Nrf2 recruitment and degradation. CRLs can target a wide range of proteins for degradation and CRL-mediated ubiquitination represents a necessary contributor of many cellular processes.

5.2 Regulation of CRL's

5.2.1 Neddylation and Activation of Cullins: Role of UBC12

CRL's are postranslationally modified by the cyclic addition and removal of the small ubiquitin-like protein (UBL), neural precursor cell expressed developmentally downregulated protein 8 (NEDD8). Neddylation is critical in many cellular functions, including cell cycle progression in mammals (Kumar, Yoshida et al. 1993, Tateishi, Omata et al. 2001), cell signaling (Zuo, Huang et al. 2013) and transcriptional stress responses (Xirodimas, Saville et al. 2004). Like ubiquitin, NEDD8 is activated, conjugated to a ligating enzyme and finally transferred to an E3 enzyme. Unlike ubiquitin, however it is proteolytically cleaved to reveal a glycine 76 residue at the C-terminal, which is necessary for the covalent attachment to substrates. NEDD8 can be cleaved by two enzymes, ubiquitin C-terminal hydrolase isozyme 3 (UCHL3) (Wada, Kito et al. 1998) and deneddylase 1 (DEN1) (Gan-Erdene, Nagamalleswari et al. 2003).

NEDD8 is activated by the NEDD8 E1 activating enzyme (NAE) in an ATP-dependent manner, forming a thioester intermediate (Walden, Podgorski et al. 2003). NAE is a heterodimer comprised of APP-BP1 and UBA3 that interacts with NEDD8 at Arg72, as mutation of this residue to alanine has revealed its necessary role for NEDD8-E1 interaction (Gong and Yeh 1999, Souphron, Waddell et al. 2008). The E1-S-NEDD8 intermediate is then transferred to a conjugating enzyme, E2. There are two known E2 enzymes, UBC12 (as known as UBE2M) and UBE2F, which bind to E3-Rbx1 and E3-Rbx2, respectively (Huang, Ayrault et al. 2009). UBC12 binds to cullin 1-4 and UBE2F binds and activates cullin 5 (Osaka, Kawasaki et al. 1998, Huang, Ayrault et al. 2009). Rbx1 and Rbx2 are RING E3 molecules that interact directly with E2 to promote the neddylation of specific lysine of cullins CTD (Zheng, Schulman et al. 2002, Petroski and Deshaies 2005). Neddylation leads to conformational changes of the CRL, which then causes activation, complex assembly and ultimately, the transfer of ubiquitin from E2 to the target molecule (Hori, Osaka et al. 1999, Podust, Brownell et al. 2000). Deneddylated cullins exist in closed conformations, which are also considered to be in an “off” state. Neddylation changes the conformation of the cullins so that they are in an open state, able to accept a substrate-bound adaptor molecule (Duda, Borg et al. 2008). In summary, neddylation is a tightly regulated and reversible process that controls CRL activity via modulation of its conformational states.

5.2.2. JAB1 and CRL Deneddylation

Neddylation is a reversible modification and deneddylation is catalyzed by a fifth member of the COP9 signalsome (CSN) also known as, Jun-activating binding domain protein 1 (JAB1 or CSN5). The CSN is a large ~450 kDa molecular weight complex that

contains eight different subunits. It was initially discovered in Arabidopsis and is highly conserved between plants and animals (Wei, Chamovitz et al. 1994). JAB1 is the most well characterized subunit of the complex and it serves many biological functions. JAB1 functions both as a member of the complex conferring its catalytic activity and can function independent of the complex (Tran, Allen et al. 2003). JAB1 was initially identified as a co-activator of the transcription, AP-1 (Claret, Hibi et al. 1996), which plays a role in a wide range of biological processes including stress response, cell growth, proliferation and apoptosis. JAB1 interacts with AP-1 to increase specificity of downstream target genes. Furthermore, given AP-1's broad function, as well as JAB1's promiscuity in interacting with other modulators of the cell cycle (i.e. p53 and p27), JAB1 upregulation is often seen in various cancers (reviewed in (Berg, Zhou et al. 2007, Shackleford and Claret 2010)). Like other constituents of the CSN, JAB1 contains the MPR1 and PAD1 N-terminal domain (MPN). JAB1's MPN is differs from other members because it contains MPN domain metalloenzyme (JAMM motif). This motif confers JAB1's isopeptidase capacity (Tran, Allen et al. 2003), which is critical in regulating CRL activity. In the absence of a substrate molecule, JAB1 can cleave NEDD8 from cullins allowing for efficient cyclic neddylation and deneddylation (Cope, Suh et al. 2002, Ambroggio, Rees et al. 2004). Mouromoto and colleagues identified JAB1's isopeptidase in the context of interferon receptor stabilization. They showed that JAB1 antagonizes the neddylation of the receptor, thereby promoting its stabilization (Muromoto, Nakajima et al. 2013). Interestingly, JAB1's function is paradoxical in nature. While deneddylation is inhibitory leading to CRL inactivity, it is necessary for its proper CRL function. Neddylation is inherently destabilizing as it

promotes autoubiquitination, however by switching off neddylation, components of the CRL are protected from degradation (Bornstein, Ganoth et al. 2006). This demonstrates JAB1's necessary role in mediating proper CRL activity, as well as stabilizing CRL components in the absence of substrate.

5.2.3 Inhibition of Neddylation-Role of CAND1

Once a CRL is deneddylated it becomes available to interact with Cullin-Associated and NEDD8-Disassociated1 (CAND1 or TIP120A). CAND1 is a negative regulator of Cul-neddylation. CAND1 is 120-kDa protein that preferentially binds to deneddylated Cullins. CAND1 is critical for CRL regulation as its binding inhibits neddylation and subsequent complex assembly (Oshikawa, Matsumoto et al. 2003, Goldenberg, Cascio et al. 2004). Like JAB1, CAND1 is critical for efficient CRL function (Schmidt, McQuary et al. 2009). CAND1's functions as a necessary CRL mediator were demonstrated *in vitro*. Ectopic overexpression of CAND1 competed with KEAP1 for CUL3 binding, which disrupts KEAP1-CUL3 interaction and induces Nrf2 stabilization. Conversely, knock down of CAND1 led also led to the stabilization of Nrf2, but via indirect effects on CRL3. While CAND1 deficiency potentiated KEAP1-CUL3 association, KEAP1's ability to efficiently target Nrf2 for ubiquitination was compromised in CAND1-deficient cells, ultimately leading to increased Nrf2 levels (Lo and Hannink 2006). In summary, CAND1 is an established negative regulator of the CRL, but also functions to maintain efficient CRL activity. Further, Nrf2 increases in the context of its loss and gain of function in the *in vitro* reflects CAND1 ability in modulating different aspects of the CRL.

5.2.3.1 Structure and Function of CAND1

CAND1 consists of 27 huntingtin-elongation-A subunit-TOR (HEAT) repeats. Each repeat is approximately 40 residues in length and are arranged such that the superhelical solenoid structure is formed. CAND1 wraps around cullins in a head-to-toe manner, in which CAND1's NTD wraps around the cullins' CTD. Therefore, CAND1's interaction with cullins extends throughout the entire length of the cullin ultimately, forming a ternary complex. CAND1 directly blocks neddylation by binding to the lysine residue critical for neddylation, (Lys720 in CUL1), effectively blocking neddylation of the scaffold. Furthermore, CAND1 also binds to the region where RING proteins dock, ultimately inhibiting their binding and maintaining the complex in a disassembled state (Goldenberg, Cascio et al. 2004). The addition of NEDD8 molecule disrupts the interaction between the cullin and CAND1, thereby displacing CAND1 (Liu, Furukawa et al. 2002).

6. DJ-1 & Regulation of Antioxidant Responses

DJ-1 is a 20-kDa multifunctional protein that is 189 amino acid in size that is encoded by the *PARK7* gene. DJ-1 was initially discovered by Nagakubo et al, as an oncogene that promotes cell growth and proliferation. This group found that co-transfection of DJ-1 with ras was sufficient to transform mouse NIH3T3 cells. DJ-1 is expressed ubiquitously by most tissues including the lung and brain, but is preferentially expressed in the testis (Nagakubo, Taira et al. 1997). Intracellular DJ-1 exists in the cytoplasm, nucleus and mitochondria (Canet-Aviles, Wilson et al. 2004). DJ-1 may also be secreted into cerebrospinal fluids of patients with Parkinson's disease (PD) (Waragai, Wei et al. 2006). While the function of DJ-1 remains largely unknown, evidence suggests that it is a redox-sensitive molecular chaperone. Its other roles include:, sensing and antagonism of oxidative stress (Ariga, Takahashi-Niki et al. 2013), male sterility (Honbou, Suzuki et al. 2003), neuroprotection (Bonifati, Rizzu et al. 2003, Yanagida, Kitamura et al. 2009), enhancing RNA-protein interactions, as well as pro-oncogenic functions (Cao, Lou et al. 2015).

6.1 Structure of DJ-1

Crystal structure of DJ-1 reveals a resemblance to heat shock protein 31 (HSP31) in bacteria (Wilson, Collins et al. 2003). Monomeric DJ-1 has a core comprised of a beta sheet, which is flanked by α -helices. In total, DJ-1 is a homodimer that consists of seven β -strands and nine α -helices (Nagakubo, Taira et al. 1997, Wilson, Collins et al. 2003).

The alpha and beta sheets are arranged to form a structure similar to that of a Rossman fold, which exists in glutamine amidotransferase (GAT) family of proteins (Horvath and Grishin 2001). DJ-1 is also structurally similar to the diverse members of the DJ-1/ThiJ/PfpI superfamily of proteins (Du, Choi et al. 2000). These families of chaperones, proteases, catalase and kinases contain a conserved ThiJ domain, whose function is largely unknown (Mizote, Tsuda et al. 1999).

It consists of three cysteine residues, Cys₄₆, Cys₅₃ and Cys₁₀₆ (Canet-Aviles, Wilson et al. 2004, Ito, Ariga et al. 2006), which confer its redox-sensing capacity. Ito et al. have found that cysteine residues Cys₄₆ and Cys₅₃ are susceptible to s-nitrosylation, while Cys₁₀₆ is not (Ito, Ariga et al. 2006). Furthermore, Cys₄₆ is critical for the dimerization of DJ-1. Although the function of dimerized DJ-1 is largely unknown, some evidence suggests that it may exist and interact with other molecules as an obligate dimer (Lee, Kim et al. 2003). The missense mutation leading to the substitution of Leu₁₆₆ for proline in DJ-1 leads to not only to its inability to form dimers, but its complete loss of function, which also has implications for the manifestation of PD (Bonifati, Rizzu et al. 2003, Moore, Zhang et al. 2003). The crystal structure solved by Lee et al. showed that dimerization of DJ-1 is facilitated by alpha helices $\alpha 1$, $\alpha 7$, $\alpha 8$ and beta sheet $\beta 4$ (Kinumi, Kimata et al. 2004). While all three cysteine residues are liable to oxidation, Cys₁₀₆ is particularly sensitive to the effects of oxidative stress (Du, Choi et al. 2000, Kinumi, Kimata et al. 2004) and is critical for the cytoprotective function of DJ-1 (Canet-Aviles, Wilson et al. 2004). Furthermore, oxidized Cys₁₀₆ is used as biomarker of PD (Saito 2014). It is oxidized to three species (Cys-SOH), cysteine sulfinic acid (Cys-SO₂H), and cysteine sulfonic acid (Cys-SO₃H). Cys-SO₂H is the most stable and confers biologically

active DJ-1. Cys₁₀₆ is the most conserved residue of DJ-1 across different DJ-1 homologues (Canet-Aviles, Wilson et al. 2004). Mutations of this residue have demonstrated its vital role in the mediation of oxidation stress. In addition to sensing cellular redox changes, DJ-1 has the capacity to attenuate oxidative stress both via Nr2 and independent of the pathway.

6.2 The Cytoprotective Roles of DJ-1

Among its many other functions, DJ-1 confers cytoprotection of different models by activating antioxidant response pathways, promoting cell survivorship, inhibiting apoptosis and maintaining mitochondrial function (Larsen, Ambrosi et al. 2011, Abdel-Aleem, Khaleel et al. 2016, Wang and Gao 2016). It is for these reasons that DJ-1 upregulation in the context of cancer is detrimental and its loss-of-function promotes the pathogenesis of PD (Hague, Rogaeva et al. 2003). In spite of the controversy regarding the roles of DJ-1, its function in oxidative stress has become widely accepted. DJ-1 loss is associated with increased sensitivity to the effects of sources of ROS (Martinat, Shendelman et al. 2004). Moreover, the converse, or overexpression of DJ-1 protects cells against the ROS-mediated apoptosis.

6.2.1 DJ-1 & the Inhibition of Oxidative Stress

DJ-1's function have been most well characterized in the context of PD, a neurological disorder that is driven by exacerbated protein misfolding and enhanced ROS (Bonifati, Rizzu et al. 2003, Bonifati, Oostra et al. 2004). In addition to the brain, DJ-1's

biological significance has been demonstrated in multiple organ systems including the lung (Bahmed, Messier et al. 2016), kidneys (Eltoweissy, Dihazi et al. 2016). The lung is highly susceptible to the effects of oxidative stress, particularly in the context of CS. Bahmed et al showed that primary alveolar type II cells isolated from heavy smokers have attenuated DJ-1 expression as compared to those isolated from non-smokers. They also that Nrf2 expression and Nrf2-dependent transcripts are restored in cells by the overexpression of DJ-1. DJ-1 regulates antioxidant responses through a number of pathways. DJ-1 has been shown to play a role in regulating oxidative stress through the induction or stabilization of Nrf2. Clements et al demonstrated that DJ-1 necessary for Nrf2 stability in non-small cell carcinoma H157, and its ablation significantly attenuates Nrf2 protein expression. Furthermore, it also decrease ARE activity, as well the Nrf2-dependent transcript, NQO1 (Clements, McNally et al. 2006). In addition to Nrf2 stability, DJ-1 induced Nrf2 activation and translocation in cardiac cells under condition of hypoxia re-oxygenation (Yan, Yang et al. 2015). While many models have demonstrated the effects of DJ-1 on Nrf2, some models have revealed that it promotes cell viability under conditions of oxidative stress independent of Nrf2 (Gan, Johnson et al. 2010, Ismail, Abdel Shakor et al. 2015).. It can potentially do so through its ability to sense and scavenge for ROS, thereby serving as redox buffer (Eltoweissy, Dihazi et al. 2016).

6.2.2 DJ-1 is an Oncogenic Protein that Inhibits Apoptotic Pathways

Oxidative stress can induce DNA damage leading to p53 activation and apoptosis. Numerous studies have demonstrated DJ-1's capacity to antagonize oxidative stress-

induced apoptosis. ASK1 is a stress responsive member of the MAPK family of proteins. ASK1 activates c-Jun N-terminal kinase and p38 signaling cascades important in stress response (Ichijo, Nishida et al. 1997). DJ-1 can inhibit ASK1 activation via direct interaction mediated by Cys₁₀₆, which is necessary for this interaction to occur as demonstrated by site directed mutagenesis (Waak, Weber et al. 2009). ASK1 activation is inhibited by the oxidoreductase protein, thioredoxin (Trx), which binds to ASK1 via its N-terminal domain. The Trx-ASK1 interaction is disrupted by presence of ROS, which oxidize Trx inhibiting its capacity to bind ASK1 (Saitoh, Nishitoh et al. 1998). DJ-1 can also indirectly inhibit the ASK1 pathway by the induction of Trx in an Nrf2-dependent transcriptional mechanism (Im, Lee et al. 2012). In addition to the ASK1 pathway, DJ-1 enhances cell viability via antioxidant upregulation and activation of pro-survival pathways, such as the Akt pathway (Yang, Gehrke et al. 2005, Tong, Katoh et al. 2006, Wang and Gao 2016). Finally, there is some evidence to implicate DJ-1's ability to negatively regulate the pro-apoptotic transcription factor, p53. DJ-1 was demonstrated to bind directly to the DNA-binding region of p53, preventing its interaction with the promoters of its downstream genes (Kato, Maita et al. 2013). Taken together, these demonstrate that DJ-1 is important in antagonizing apoptosis via direct effects on pro and anti-apoptotic signaling cascades, as well as the upregulation of antioxidant responses.

Chapter 2

Methods & Materials

2.1 Experimental Models

2.1.1 Animal Models

All animal protocols were conducted as approved by the Johns Hopkins University Institutional Animal Care and Use Committee. MIF knockout were generated in a C57BL/6 as described in (Bozza, Satoskar et al. 1999). Wild type and MIF deficient 8-10 week old mice were exposed to filtered air or CS. Mice were exposed to CS for 5 hours/day, 5 days/week for either 3 days (acute) or 6 months (chronic). CS exposure was completed in the manner previously described (Sussan, Rangasamy et al. 2009). CS exposure was achieved by using a smoke machine (Model TE-10, Teague Enterprises) that burned 2R4F reference cigarettes (Tobacco Research Institute, University of Kentucky). The machine was adjusted to produce a mixture of side stream and mainstream smoke also as previously described (Rangasamy, Cho et al. 2004).

2.1.2 In vitro models & Treatments

Human microvascular pulmonary endothelial cells were purchased from Lonza (HMVEC-L, CC2527, Lot number: 000441097, Lonza, MD) and were maintained in endothelial growth medium (EGM-2, Lonza, MD) supplemented with EGM-MV Bullet Kit (Lonza, MD). Cells were utilized for experiments from passages 4 to 9. Rat pulmonary microvascular endothelial cell (RMVEC-L, RA6011, Cell Biologics, IL) were maintained in complete endothelial cell medium (Cell Biologics, IL) from passages 4 to 9. Cell cultures were maintained at 37°C and 5% CO₂. Cigarette smoke extract (CSE) was prepared as described in (Damico, Simms et al. 2011) in a 20% solution (v/v) in

complete media. In our recombinant MIF (rMIF) treatment experiments, HMVEC-L and RMVEC cells were treated with recombinant MIF (rMIF, (#289-MF (human) and 1978-MF (mouse), respectively R&D Systems, Minneapolis, MN) was dissolved in 0.1% bovine serum albumin in PBS. Cells were treated with a final concentration of 100ng/mL. The ROS-sensitive dye, 2',7'-dichlorodihydrofluorescein (H₂DFCDA, D-399, Life Technologies, Carlsbad, CA) was utilized to assess intracellular ROS. HMVECs were incubated with H₂DFCDA for 45 mins, then treated with either, vehicle (0.1% BSA), rMIF (100ng/mL) or N-acetylcysteine (2mM) after CSE exposure (20% v/v). H₂DFCDA becomes oxidized into a fluorescent DCF indicator of ROS which was measured every 5 minutes (495/520nm). Delta fluorescence is defined as the ratio of post-stimulus fluorescence over baseline.

2.2 RNA Isolation & RT-PCR

Total RNA was isolated from lung homogenized in TRIzol (Invitrogen/ Life Technologies, Carlsbad, CA). RNeasy kit (Qiagen, Valencia, CA) was then used to further purify the RNA. Purified RNA was then reverse transcribed into complementary DNA (cDNA) by RT2 FirstStrand cDNA kit (SABioscience/ Qiagen, Valencia, CA). SYBR Green Dye (Qiagen, Valencia, CA) based Real-Time Quantitative PCR was completed to measure gene expression of NADPH dehydrogenase quinone 1 (NQO1) (5' AGCCAATCAGCGTTCGGTAT-3', 5'-GTAGTTGAATGATGTCTTCTCTGAAT-3'), glutathione peroxidase 2 (GPX2) (5'-CCCTTCCGTCGCTACAGCCG-3', 5'-TCCCAGGGTCTCCCGAGGGTA-3') beta actin (5'ACGGCTCCGGCATGTGCAAA-3', 5'-ACCATCACACCCTGGTGCCT-3') (IDT San Jose, CA).

2.3 Western Blot

For protein isolated from tissues, lungs were washed cold PBS and homogenized using tissue lysis beads (Next Advance, NY, USA; PINKE5) in 1x cell lysis buffer (Cell Signaling, Boston, MA) that contained proteinase inhibitor cocktail and other inhibitors (Sigma Chemical Co., St. Louis, MO; P8340). For protein-derived from cells, media was aspirated from wells and cells were rinsed with PBS. Cells were then lysed with 1x cell lysis buffer. All protein lysates were centrifuged and quantified with BCA assay (Thermo Scientific, Waltham, MA). Proteins were then separated by an SDS-PAGE gel (Thermo Scientific, Waltham, MA) and electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore Corp., Billerica, MA). After blocking for at least 1 hour in 5% dry milk, proteins were detected by the following antibodies: actin-HRP (#5125), JAB1 (#6895), cullin 3 (#2759), Myc-tag (#2276), HA-Tag (#3724), DJ-1 (5933) were all purchased from Cell Signaling (Boston, MA) and MIF (#20121, Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were then incubated with secondary antibody conjugated to HRP (#5571, Cell Signaling, Boston, MA). Chemiluminescent substrate was then used to detect the protein-antibody complexes (ECL; Amersham Pharmacia Biotech, Piscataway, NJ).

2.4 Plasmid Preparation & Isolation

Ampicillin-resistant *E. coli* containing pcDNA, Nrf2-myc, JAB1-myc, UBC12-myc, dnUBC12, CAND1-myc, DJ-1-myc were spread on agar one bacterial colony was then selected and expanded in ampicillin LB broth. Bacteria was then centrifuged and

pelleted within 24 hours of inoculation and plasmids were purified using a Maxi-prep kit (Qiagen, Valencia, CA). DNA sequence was subsequently confirmed. pcDNA3-FLAG-HA was a gift from William Sellers (Addgene plasmid #10792), pcDNA-Myc3-Nrf2 and pcDNA-Myc3-CAND1 were gifts from Yue Xiong (Addgene plasmid #21555 and #19948). FLAG-HA-COPS5 was a gift from Wade Harper (Addgene plasmid #22541), pGW1-Myc3-DJ-1 was gifted from Mark Cookson (Addgene plasmid #29347), UBC12-HA was a gift from Rachel Klevit (Addgene plasmid #31423) and pcDNA-HA-dnUBC12 were all obtained from (Addgene, Cambridge, MA)

2.5 Transfections

2.5.1 Transfection of siRNA

For gene-silencing experiments, cells were about 80-90% confluent and siRNA knockdowns of JAB1 and DJ-1 (GE Dharmacon, Lafayette, CO) were achieved using Geneporter 2 lipid-based transfection reagent (Genlantis, San Diego, CA). Complete media was removed and serum-free media was used to for transfections. Complete media was added 4 hours post-transfection. Media containing transfection reagent was removed and complete media was added 24 hours post-transfection. Cells were harvested with 1x cell lysis buffer (Cell Signal Technology, Danvers, MA) 48 hours and 72 hours post-transfections to ensure significant of JAB1 and DJ-1 knockdowns, respectively.

2.5.2 Plasmid Transfection

Cells were plated to achieve 70-80% confluency within 24 hours of plating and overexpression of plasmids was completed in serum-free media by the lipid-based

transfection reagent, Lipofectamin 2000 (Thermo Scientific, Waltham, MA). Cells were harvested within 24 hours for analysis of ectopically expressed proteins. In Nrf2 stability experiments, cells were treated rMIF (100ng/mL) for 24 hours and with and without the protease inhibitor, 50 μ M MG-132 (Cell Signal Technology, Danvers, MA) for 1.5 hours.

2.6 Dual Luciferase Assay

Cells were transfected with 1 μ g of a mixture of an Nrf2-responsive luciferase construct under the control of the antioxidant response element (ARE) and a constitutively expressing renilla construct, that served as an internal transfection control in a (40:1) using Lipofectamin 2000 as described above. For rMIF treatment experiments, cells were treated with 100ng/mL of rMIF within 24 hours post-transfection and harvested at 0, 12 and 24 hours with passive lysis buffer. For co-transfection experiments, the total amount of DNA transfected into cells on a 6-well plate was maintained at 1 μ g by co-transfecting about one-third of the ARE-luciferase construct with remaining alternative plasmid. To ensure complete lysis, lysates were subject to one freeze-thaw cycle. Luciferase assays were completed in white opaque 96-well plates as outlined by the manufacturer of the Dual-Luciferase Reporter Assay System (E1910 Promega, Madison, WI) and luciferase activity was measured by using a luminometer.

2.7 Caspase 3 Activity Assay

For the DJ-1 knockdown experiment, cells were transfected with DJ-1 siRNA, as described in section 2.5 and treated with CSE 72 hours post-transfection for 0, 2, 4, 6 and 8 hours. Cells were then harvested with diluted 1x cell lysis buffer and probed for

caspase3 activity using the Caspase-Glo 3/7 assay (G8091, Promega, Madison, WI) according to manufacturer's protocol. The caspase substrate was reconstituted in buffer prior to use and equal volumes of cell lysates and caspase 3 buffer were added in a white opaque 96-well plate. The cleavage of the substrate by the caspase produces a luminescent signal that is measured by a luminometer. All luminescent data were then normalized to cell lysate concentrations, determined via a bicinchoninic acid (BCA) assay.

2.8 Statistical Analysis

For comparisons among groups of normally distributed data sets, Student's t test or 2-way ANOVA with post hoc Bonferroni correction was used.

Chapter 3

The Effects of MIF on the Nrf2-Antioxidant Response Pathway

3.1 Abstract

CS is the leading cause of, emphysema, a major type of COPD that is characterized by enlargement of the airspaces distal to the terminal bronchioles, permanent destruction of alveolar walls, and airflow limitation, ultimately leading to gas exchange impairment (Rodgers, Ezzati et al. 2004). CS contains over 4,000 chemicals and many of its toxic ingredients include carcinogens, unstable free radicals, particulate matter and volatile organic compounds (Talhout, Schulz et al. 2011). These chemicals contribute to increased ROS production, DNA damage and ultimately lead to EC apoptosis (Damico, Simms et al. 2011, Fallica, Varela et al. 2016). Under conditions of oxidative stress, such as in the case of CS exposure, cells respond by up-regulating proteins that scavenge and inhibit the effects of excess pro-oxidants. Nrf2 is a transcription factor that binds to the ARE and upregulates the transcription of many antioxidant genes that work to scavenge and neutralize ROS (Itoh, Chiba et al. 1997). There is evidence that the antioxidant pathway is compromised in COPD, leading to diminished antioxidant capacity and disease progression, implicating Nrf2's necessary role in preventing oxidant-mediated lung disease (Suzuki, Betsuyaku et al. 2008, Zhao, Eguchi et al. 2017).

MIF is a pleiotropic cytokine that has been shown to antagonize CS-induced EC apoptosis *in vitro* (Damico, Simms et al. 2011) and *in vivo* (Fallia, Varela et al. 2016). MIF's has been implicated in positively regulating antioxidant responses the context of oxidant-mediated injury. Aged MIF^{-/-} mice have increased susceptibility to radiation-induced lung injury (RILI) and associated decreased Nrf2-expression and antioxidant

responses to ROS (Mathew, Jacobson et al. 2013). We used *in vitro* and *in vivo* models to test the hypothesis that MIF is a regulator of antioxidant responses during CS exposure. We have shown that exogenous recombinant MIF protein (rMIF) is sufficient to attenuate ROS accumulation in primary human microvascular endothelial (HMVECs) cells after CS exposure. Further, recombinant rMIF is sufficient to drive both Nrf2 protein expression and ARE activity in both HMVECs and rat microvascular endothelial cells (RMVECs). We also demonstrated MIF is a necessary mediator of antioxidant responses in the context of CS-induced oxidative stress *in vivo*. MIF deficient (MIF^{-/-}) mice are unable to upregulate Nrf2-dependent transcription in response to acute CS-exposure. Taken together, we show that MIF is a positive regulator of Nrf2, which confers its ability to mediate antioxidant responses during CS-induced oxidative stress.

3.2 Introduction

Cells are constantly subject to various sources of ROS and other forms of pro-oxidants. Endogenous ROS sources are the by-products of normal physiological mitochondrial metabolism (Barrett, DeGnore et al. 1999, Marnett 1999, Valko, Rhodes et al. 2006, Valko, Leibfritz et al. 2007). Cells are also susceptible to exogenous sources of ROS, which due to exposures to heavy metals (Lodovici and Bigagli 2011, Jan, Azam et al. 2015), components in CS (Asami, Manabe et al. 1997, Valavanidis, Vlachogianni et al. 2009) and particulate matter (Lodovici and Bigagli 2011). Increased exposure to pro-oxidants can perturb the sensitive redox state of the cell. They are therefore equipped with mechanisms to combat the effects of these pro-oxidant exposures through the upregulation of antioxidants (Nguyen, Nioi et al. 2009). Oxidative stress is caused by the imbalance between the production of oxidants and the cell's capacity to detoxify and neutralize them. While some endogenous sources of oxidants are important in cell signaling pathways (Banki, Hutter et al. 1999, Barrett, DeGnore et al. 1999), excessive presence of oxidants can cause damage to biological macromolecules (Thannickal and Fanburg 2000). Oxidative stress leads to DNA damage via strand breaks and promotion of adduct formation. It also causes lipid peroxidation (Wills 1966) and oxidation of proteins, which can ultimately lead inappropriate DNA damage repair or cell death (Pero, Roush et al. 1990, Asami, Manabe et al. 1997). This imbalance between ROS and antioxidants can have many detrimental effects on cell biology, eventually leading to chronic inflammation and disease.

COPD is an irreversible lung disease that is characterized by the progressive chronic obstruction of the airways that interferes with normal breathing. COPD is the result of the complex interplay between genetics and the environmental irritants (reviewed in (Mannino and Buist 2007)). One established risk factor is the lifetime toxic burden associated with the chronic inhalation of noxious oxidants and particles (Kirkham, Caramori et al. 2011). In the United States, chronic exposure to CS is the leading cause of emphysema, a major type of COPD (Ezzati and Lopez 2004), contributing to about 80-90% of all cases (Mannino and Buist 2007). Emphysema is a type of COPD that is characterized by irreversible enlargement of airspaces distal to the terminal bronchiole and progressive airflow limitation (Hogg, Chu et al. 2004). This enlargement is due to increased alveolar wall destruction, which markedly reduces the surface area for adequate gas exchange (Hogg 2004). Links between markers of oxidative stress and severity of COPD have been previously established. Kluchova et al. showed that the severity of COPD is associated with increased levels of the lipid peroxidation product, malondialdehyde (MDA) (Rahman 2005, Kluchova, Petrasova et al. 2007). Furthermore, CS-induced COPD was correlated with enhanced DNA damage as well as oxidation of proteins resulting in the formation of protein carbonyl groups (Ceylan, Kocyigit et al. 2006).

The transcription factor and master regulator of the anti-oxidant response pathway, Nrf2 (Itoh, Chiba et al. 1997, Chan and Kan 1999, Itoh, Ishii et al. 1999) is responsible for the control of over 200 antioxidants (Kobayashi and Yamamoto 2006). Once activated, Nrf2 translocates to the nucleus where it binds to the ARE, located in the promoter of many antioxidant genes. The protective role of the Nrf2 antioxidant response

pathway has been well characterized in the context of the lung. Many studies have reported the protective role of the Nrf2-antioxidant pathway in the context of ROS-induced injury in the context of the lung (reviewed in (Boutten, Goven et al. 2011)). They have also shown that it antagonizes the effects of CS exposure, an established determinant of COPD. The importance of Nrf2 in the setting of CS-induced emphysema was demonstrated by Rangasamy and colleagues, who showed that emphysematous tissue remodeling is exacerbated in Nrf2 deficient mice (Nrf2^{-/-}). They also showed that Nrf2^{-/-} mice have increased oxidative stress markers, inflammation, EC and EpiC apoptosis after chronic exposure to CS for 6 months (Rangasamy, Cho et al. 2004). Interestingly, Nrf2 expression is also altered with chronic smoke. Its expression is attenuated in lavaged macrophages obtained from aged smokers as compared to non-smoking age-matched individuals, suggesting that chronic exposure to CS downregulates Nrf2 in the aging lung (Suzuki, Betsuyaku et al. 2008), thereby conferring greater oxidative injury.

The lung microvasculature is critical for maintaining homeostasis, gas exchange and proper function of the alveolar septa (Sirianni, Chu et al. 2003). ECs are critical for the integrity of the capillary network as well as alveolar structure (Le Bot, 2015). EC apoptosis has been demonstrated to be a necessary and sufficient mechanism that drives emphysematous tissue remodeling (Kasahara, Tuder et al. 2000, Tuder, Zhen et al. 2003, Gordon, Gudi et al. 2011). Specifically targeting EC for apoptosis by either a peptide-directed method or via the pharmacological inhibition of the vascular endothelial growth factor (VEGF) receptor is sufficient to drive emphysematous tissue destruction in rodent models (Kasahara, Tuder et al. 2000, Giordano, Lahdenranta et al. 2008). Circulating

endothelial microparticles (EMPs), which are markers of EC apoptosis are elevated in individuals with COPD with normal lung function. This implies that EC apoptosis is an early phenomenon that occurs prior to emphysematous tissue remodeling and pulmonary dysfunction (Gordon, Gudi et al. 2011). Therefore, elucidating the molecular mechanisms that regulate EC apoptosis in the context of CS exposure provides important insight into the pathogenesis of emphysema and potential insight into the well-recognized clinical heterogeneous responses to smoke exposure in humans.

While MIF is long recognized as an important cytokine that regulates the innate immune system, our understanding of its molecular and cell biologic properties have expanded substantially in more recent years. It is elevated under conditions of inflammation, its functions have been linked to promoting cell survivorship and inhibiting apoptosis (Wistow, Shaughnessy et al. 1993, Fingerle-Rowson, Petrenko et al. 2003). Fallica et al showed that MIF is altered in patients with COPD. Serum levels of MIF are decreased in patients with advanced COPD as compared to non-smokers. Further stratifying the COPD cohort revealed that MIF is markedly reduced in patients with advanced stage COPD. Thus, reduction in circulating MIF is associated with worse disease in humans (Fallica, Boyer et al. 2014). Similar findings were observed in a second independent cohort, which showed that MIF is not only deficient in patients but also declines in the BAL of aged mice exposed to chronic CS (Sauler, Leng et al. 2014).. Chronic CS exposure also decreases MIF expression in the lung tissue of mice, suggesting CS directly or indirectly antagonizes MIF expression *in vivo*.. Furthermore, MIF deficiency in chronic CS exposure results in increased ROS, markers of DNA damage, and EC apoptosis, ultimately driving emphysematous tissue damage (Fallica,

Boyer et al. 2014, Sauler, Leng et al. 2014, Fallica, Varela et al. 2016). This demonstrates a critical role for MIF in antagonizing ROS-mediated injury in the lung. The observed increases in sensitivity of pulmonary ECs in the setting of MIF deficiency *in vivo*, suggests that this cell type is particularly dependent on MIF in the context of CS exposure. Similar studies demonstrate that knockout of either MIF or one of its receptor, CD74, potentiates spontaneous emphysematous tissue remodeling in aging mice (Sauler, Leng et al. 2014) implicating MIF's role in maintaining lung homeostasis during aging, although the molecular mechanism(s) of cytoprotection in the aging model remain to be defined experimentally. A role for MIF in cytoprotecting against ROS-mediated injury in other injury models in the lung and in other organs have also been demonstrated. Studies of MIF knockout mice demonstrate a protective role for MIF in radiation-induced lung injury, a model characterized by increased markers of oxidative injury. MIF^{-/-} mice had increased BAL protein-a marker of lung permeability, enhanced histological markers of pulmonary inflammation and attenuated antioxidant capacity (Mathew, Jacobson et al. 2013). Aged MIF^{-/-} mice exposed to ionizing radiation-induced disease phenotype was rescued via the administration of the antioxidants, implicating that injury is driven by oxidative stress and that MIF is a necessary mediator normal of antioxidant defenses (Mathew, Jacobson et al. 2013). In another ROS-mediated injury model of ischemic-reperfusion within the heart, MIF^{-/-} mice have enhanced cardiac dysfunction associated with increased activation of the stressed-responsive kinase, JNK and its downstream target, BCL2-associated agonist of cell death (BAD) (Qi, Hu et al. 2009). In contrast to the work in the heart, MIF-mediated antagonism of another stress-response kinase p38 MAPK, not JNK, has been linked to its ability to antagonize CS-induced injury *in vitro*

and *in vivo* (Fallica, Boyer et al. 2014, Fallica, Varela et al. 2016). There is a growing body of literature that characterizes MIF's role is cytoprotective in models of injury characterized by increase ROS, yet the molecular mechanisms responsible for its biological properties remain to be defined.

Given Nrf2's critical role in inducing enzymes important in the detoxification of oxidants and the expanding data that MIF antagonizes ROS-mediated injury including CS-induced emphysema, we postulated that MIF plays a role in mediating antioxidant responses in the context of CS (Qi, Hu et al. 2009, Fallica, Boyer et al. 2014, Sauler, Leng et al. 2014, Fallica, Varela et al. 2016). Taken together, we hypothesize that MIF is inhibiting oxidant-mediated injury, in part, through its capacity to induce antioxidant responses. We therefore utilized *in vitro* and *in vivo* models to understand MIF's effects on ROS production, Nrf2 activity and protein expression and Nrf2-dependent responses. Here we provided data that MIF is required for optimal antioxidant responses in the lung context of CS exposure and is sufficient to positively regulate Nrf2 via a proteasome-dependent mechanism.

3.3 Results

3.3.1 MIF is Necessary for CS-Induced Antioxidant Transcripts in the Lung *in vivo*

It is well-established that CS, a composite of over 4,000 chemicals, induces ROS production in animal and cell models (Church and Pryor 1985). Chronic CS exposure also significantly blunts MIF expression in the lungs of mice chronically exposed to CS (Fallica, Boyer et al. 2014, Sauler, Leng et al. 2014). Furthermore, MIF loss is associated

with an increase in ROS accumulation in the lung, as well as increased emphysematous tissue remodeling (Fallica, Varela et al. 2016). Given MIF's role in antagonizing ROS *in vivo* and as well oxidant-mediated injury, we predict that MIF antagonizes ROS accumulation through its effects on antioxidant and detoxifying genes.

In order to test this, we exposed MIF^{+/+} or MIF^{-/-} mice for 3 days with either filtered air or CS as previously describe (Fallica, Varela et al. 2016). Next, we utilized quantitative RT-PCR (qPCR) to characterize the effects on antioxidant gene expression in total RNA isolates from whole lung lysates. NAD(P)H Quinone Dehydrogenase 1 (NQO1) and Glutathione Peroxidase 2 (GPX2) are two established Nrf2-dependent antioxidant genes. The expression of each transcript increased 2 and 7-fold, respectively in MIF^{+/+} exposed to CS compared to their air-exposed counterparts ($p < 0.01$) (Fig. 3-1A and 3-1B). CS exposure failed to induced these transcripts in MIF^{-/-} mice despite the fact that these animals have increased lung ROS accumulation (data not show, reference JON). This implicates MIF as a necessary mediator of Nrf2-dependent responses during CS-exposure *in vivo* (n=3) (Fig 3-1A and 3-1B).

3.3.2 Recombinant MIF (rMIF) Decreases CS-Induced ROS Accumulation *in vitro*

MIF deficiency potentiates ROS and ROS-driven injury (Mathew, Jacobson et al. 2013, Fallica, Varela et al. 2016), which is associated with the failure to express Nrf2-dependent antioxidant transcripts (Mathew, Jacobson et al. 2013). This supports a model in which MIF inhibits ROS-mediated injury by promoting antioxidant gene expression *in vivo*. To determine if MIF is sufficient to alter ROS accumulation, we used an *in vitro* model.

We therefore, sought to investigate if exogenous MIF was sufficient to blunt the effects of CS-induced ROS accumulation in HMVECs. We utilized fluorogenic ROS-sensitive dye, 2',7'-dichlorofluorescein diacetate (DCFDA), which becomes oxidized to the fluorescent marker, DCF in the presence of ROS. Based on previously published evidence on the efficacy of rMIF in protecting HMVEC's from CS-induced apoptosis, we utilized a dose of 100ng/mL (Damico, Simms et al. 2011). Cells were pre-treated with vehicle control (0.1% bovine albumin serum (BSA) in PBS), rMIF (100ng/mL) or N-acetyl cysteine (NAC), an antioxidant that served as a positive control for this study, 4 hours. We then challenged cells with cigarette smoke extract (CSE) at a concentration 2×10^{-2} cigarette per 1 mL of PBS to generate 20% (v/v) as previously described in Chapter 2. This dose was chosen based on prior dose-response curves for CSE-induced EC apoptosis. Fluorescence was assessed at baseline and after exposure to CSE and reported as the change in fluorescence over baseline ($\Delta F/F_0$) of each biological sample. CSE induced over 1.2-fold increase in ROS accumulation in HMVECs in cells treated with the BSA control. Pre-treatment of cells with rMIF significantly antagonized intracellular ROS, implicating its role in inhibiting CS-induced ROS production in ECs. NAC treatment suppressed the change in DCF fluorescence, demonstrating specificity (Fig 3-2). Thus, exogenous MIF had the capacity to blunt CSE-induced intracellular ROS accumulation in EC in vitro.

3.3.3 MIF Drives ARE Activity in Pulmonary ECs

MIF antagonizes the accumulation of CSE-induced ROS, implicating MIF as a negative regulator of CSE-induced oxidant formation. We have also previously observed

that MIF deficiency *in vivo* potentiates CS-induced ROS in the lung (Fallica, Boyer et al. 2014), which is associated with a significant attenuation in antioxidant gene expression (Fig 3-1A & 3-1B). Thus, we predict that MIF directly or indirectly promotes antioxidant gene expression accounting for its effects on ROS concentrations. Therefore, then sought to identify the effects of MIF directly on the ARE, a DNA element within the promoter of many antioxidant genes responsive to the Nrf2 transcription factor.

We transfected HMVEC's with a dual ARE reporter system, which is a mixture comprised of an ARE-responsive firefly luciferase construct and a Renilla construct under the control of the constitutive active CMV promoter, in a 40:1 ratio. Renilla served as an internal transfection control. After about 24 hours post-transfection, we treated cells with rMIF for 0, 12 and 24 hours (Fig. 3-2a). ARE activity was then determined by a luciferase assay that was performed under the manufacturer's protocol. We found that treatment with rMIF significantly drives ARE-activity by about 1.5 fold 24 hours post-treatment ($p<0.01$). Given this data, MIF's ability to drive antioxidant responses, as demonstrated by other models, may be through its ability to positively regulate ARE activity and downstream antioxidant gene transcription.

3.3.4 MIF is Necessary for Optimal ARE Activity Under Conditions of Oxidative Stress

We have demonstrated that MIF is able to inhibit CS-mediated ROS accumulation both *in vivo* and *in vitro* and have postulated that it may antagonize ROS propagation via its capacity to induce antioxidant responses. We therefore studied the effects of exogenous MIF on ARE activity and found that it positively regulates its activity. We

then aim to understand if MIF is a necessary mediator of CS-induced ARE activity. In other words, we ask if the loss of MIF would blunt CS-induced ARE activity.

In order to address this question of necessity, we utilized MIF siRNA to abrogate MIF *in vitro*. We co-transfected cells with either on-target control (OT) siRNA or MIF siRNA and the same dual ARE luciferase constructs (as described previously). Cells were then treated with vehicle control 20% PBS (v/v) or cigarette smoke extract (CSE) (2x10⁻² cigarette/mL) 24 hours post-transfection. While we have successfully knocked down MIF (data not shown), we found no difference in basal ARE activity in cells that were transfected with OT siRNA or MIF siRNA, suggesting that MIF is not necessary mediator of ARE under basal conditions. CSE was sufficient to significantly drive ARE activity by nearly 9-fold in cells co-transfected with OT. The ARE activity response to CSE was significantly blunted in cells that were transfected with MIF siRNA, demonstrating, MIF as a necessary mediator of optimal CSE-induced ARE activity in EC *in vitro* ($p=0.057$) (Fig. 3-3).

3.3.5 rMIF Promotes Stabilization of Nrf2 in a Proteasome-Dependent Manner

Provided that we have demonstrated that MIF is both a necessary and sufficient regulator of ARE activity *in vitro* and that Nrf2 is the master regulator of the ARE, we proceeded to investigate the effects of MIF on Nrf2 expression. We predicted that MIF is enhancing antioxidant responses via its capacity to positively regulate Nrf2. In order to test the hypothesis that exogenous MIF is impacting Nrf2 protein expression, we treated HMVECs with either vehicle control (0.1% BSA) or rMIF (100ng/mL). Cells were then lysed 24 post-treatment, which was the amount of time required to significantly drive

ARE activity. Endogenous Nrf2 expression was evaluated by western blotting whole cell lysates. Results reveal that rMIF was sufficient to significantly increase Nrf2 protein expression by approximately 2-fold (Fig. 3-4).

While we have demonstrated that MIF increases Nrf2 protein expression, it is unclear if MIF is doing so by upregulating its expression or by impeding its degradation. Nrf2 is a tightly regulated molecule with a high rate of turnover. Under basal conditions, Nrf2 interacts with the redox-sensitive molecule KEAP1, which adapts Nrf2 to the cullin-RING ligase complex (CRL3), machinery required to target it for ubiquitination and subsequently, degradation by proteasome (Itoh, Chiba et al. 1997). We therefore, hypothesize that MIF is inducing Nrf2 stability by interfering with its proteasomal-mediated degradation. To test this, we over-expressed myc-tagged Nrf2 under the influence of the constitutively expressed CMV promoter in RMVECs using transient transfection. This allowed us to overcome technical limitations inherent in detecting endogenous Nrf2 with the commercially available antibodies in these cells. Further, it eliminated any effect of MIF on the endogenous Nrf2 promoter. Ectopic Nrf2 protein was detected with the myc- epitope tag. Twenty hours post-transfection, we treated cells with either 0.1% BSA (vehicle (Veh) control) or rMIF (100ng/mL). In order to investigate the effects of MIF on the proteasomal-mediated degradation of Nrf2, we utilized an established chemical inhibitor of the proteasome, MG-132. MG-132 inhibits the proteasome-mediated degradation, thereby allowing for the accumulation of Nrf2. About 24 hours post-treatment of either Veh or rMIF, we then treated cells with either, the vehicle control, DMSO or MG-132 (50 μ M) for an additional 90 minutes. This dose and time have been extrapolated from previous experiments and been shown to effectively

block the proteasome (data not shown). Cells were lysed 24 hours post-treatment and whole cell lysate were analyzed by western blot for ectopic myc expression. We found that rMIF treatment significantly upregulated Nrf2 expression by approximately, 3-fold as compared to Veh control in the DMSO-treated arm ($p<0.01$). As expected, the inhibition of the proteasome by the chemical, MG-132 was also sufficient to drive Nrf2 expression by a similar increase (Veh+MG-132) ($p<0.01$). Interestingly, the treatment of both rMIF and MG-132 did not potentiate a further increase in Nrf2 expression when compared to either the rMIF+DMSO and Veh+MG-132 arms, implicating MIF's ability to increase Nrf2 expression is mediated through a proteasome-dependent mechanism (Fig. 4-5). These provide evidence for MIF's ability to enhance Nrf2 expression by impeding its degradation.

3.4 Discussion & Conclusion

Maintenance of redox status within the cell is critical to its survival. Oxidative stress is defined as a state in which the amount of oxidants and pro-oxidants overwhelm the antioxidant capacity in a cell, thereby leading to changes in the redox status and damage to macromolecules (Wills 1966). In order to combat the effects of excess ROS, cells express Nrf2, a transcription factor critical in regulating anti-oxidant gene expression (Nguyen, Nioi et al. 2009). Our data provides evidence for the role of the cytokine, MIF in mediating antioxidant responses in the context of CS-induced oxidative stress.

Chronic CS exposure remains to be a leading risk factor in developing COPD in the United States, attributing to approximately 80-90% of cases (Sethi and Rochester 2000). COPD is a heterogeneous disease and one of the established contributors of disease pathogenesis is oxidant and antioxidant imbalance. Patients with COPD have not only increased markers of inflammation, that correlate with disease severity but they also have a greater imbalance between oxidant and antioxidants, which results in enhanced oxidant burden in patients with COPD (Marsh, Zaiser et al. 2017). In addition to an imbalance of oxidants and antioxidant, there is a growing body of literature that demonstrates Nrf2's role in protecting the lung from pathogenesis of the COPD (Cho, Reddy et al. 2006). The loss of Nrf2 was shown to drive emphysematous tissue remodeling in mice chronically exposed to CS (Iizuka, Ishii et al. 2005). Furthermore, Nrf2 expression is attenuated, while KEAP1 expression is significantly increased in alveolar macrophages isolated from patients with emphysema as compared to those without disease (Goven, Boutten et al. 2008). We provide evidence that MIF is a necessary mediator of Nrf2 responses in the context of CS exposure. Acute exposure to CS induces the Nrf2-dependent genes, NQO1 and GPX2 in wild-type mice. This induction is lost however, in MIF-deficient mice, implicating MIF as a necessary mediator of antioxidant responses in the context of CS.

Emphysema, a major type of COPD is characterized by permanent enlargement of the alveolar walls. EC apoptosis has been shown to be both a necessary sufficient mechanism to induce emphysematous tissue remodeling (Yokohori, Aoshiba et al. 2004, Demedts, Demoor et al. 2006). Segura-Valdez and colleagues showed that EC apoptosis was elevated in the lungs of patients with COPD as compared to their controls (Segura-Valdez, Pardo et al. 2000). Furthermore, Giordano et al showed that specifically targeting

ECs for apoptosis was sufficient to induce emphysematous tissue remodeling in mice independent of CS exposure (Giordano, Lahdenranta et al. 2008). Clinical data shows that circulating EMPs, indicative of EC apoptosis is an early phenomenon that predates emphysematous remodeling. MIF has been proven to be an important in maintain EC homeostasis by antagonizing p53-dependent apoptosis in the context of CS (Damico, Simms et al. 2011). Furthermore, its loss is associated with increased ROS production and ROS-induced injury (Fallica, Boyer et al. 2014). Here, we provide evidence that one of the mechanisms by which MIF is conferring cytoprotection is through its capacity to impact Nrf2 stability and mediate antioxidant responses.

Previous studies have shown that MIF is altered in patients with COPD and that MIF is significantly depressed in advanced-stage COPD (Sauler, Leng et al. 2014, Fallica, Varela et al. 2016). It remains uncertain if this phenomenon is causative or the result of disease in humans. MIF however, is a necessary inhibitor of emphysematous tissue remodeling in a well-established preclinical model of CS-induced lung injury, as MIF^{-/-} mice have increased emphysematous tissue remodeling after chronic exposure to CS (Fallica, Boyer et al. 2014). Furthermore, MIF^{-/-} mice also have increased activity of the endothelial ROS-producing enzyme, xanthine oxidoreductase (XOR), which was accompanied by an increase in ROS production in the lung tissues (Fallica, Varela et al. 2016). These findings establish the importance of MIF in inhibiting not only emphysematous tissue remodeling, but also in attenuating ROS production *in vivo*. Given evidence of MIF-suppressed ROS production, we sought to determine MIF's effects on antioxidant responses. We found that pre-incubation of HMVECs was sufficient to blunt

ROS accumulation after CSE exposure, implicating its role in blunting ROS production both *in vivo* (Fallica, Varela et al. 2016) and *in vitro* (Fig. 3-1).

There is sufficient evidence to suggest that MIF is impacting the redox statues of the cell, we therefore asked if MIF is impacting the antioxidant response element activity. The ARE is a redox sensitive *cis*-acting DNA element located in the promoter region of many antioxidant genes (Rushmore, Morton et al. 1991). We demonstrated that rMIF was sufficient to induce ARE activity within 24 hours of EC treatment, independent of CSE exposure (Fig. 3-3b). Furthermore, we also showed the abrogation of MIF *in vitro* by siRNA, was sufficient to attenuate CS-induced ARE activity, implicating MIF's necessary role in mediating optimal ARE activity under conditions of oxidative stress (Fig. 3-3). The ARE is primarily regulated by Nrf2 (Moi, Chan et al. 1994), we therefore postulated that MIF is impacting the ARE via its capacity to impact Nrf2 expression. We found that treatment of ECs with rMIF for 24 hours was sufficient to increase endogenous Nrf2 protein expression (Fig. 3-3a). This suggests that MIF is impacting Nrf2 expression potentially via its capacity to either increase its expression or impede its degradation.

Nrf2 controls the expression of many genes, making its tight regulation crucial for normal cell function. Nrf2 is negatively regulated by KEAP1, which serves to adapt Nrf2 to the ubiquitination machinery and target it for proteasomal-mediated degradation (Taguchi, Motohashi et al. 2011). In order to elucidate the mechanism by which MIF is impacting Nrf2 expression, we investigated the effects of the proteasome on MIF-induced Nrf2 expression. By chemically inhibiting the proteasome with the drug MG-132, we provide evidence that MIF is increasing Nrf2 in a proteasomal-dependent mechanism in

Nrf2-myc transfected RMVECs. We show for the first time that MIF is impacting Nrf2 expression at the level of protein degradation. This may be achieved through MIF's capacity to either impact either KEAP1 or a component of the ubiquitination machinery, Cullin-RING ligase 3 (CRL3). It has been established that MIF binds to c-Jun activation domain binding protein-1 (JAB1)/subunit 5 of the COP9 signalosome (CSN5) and inhibits its ability to activate downstream signaling pathways (Kleemann, Hausser et al. 2000). JAB1 has isopeptidase activity that serves to remove a ubiquitin-like molecule (NEDD8), which ultimately regulates the activity of the KEAP1-CUL3 ligase complex (Cope, Suh et al. 2002). Given that JAB1 interacts with and negatively regulated by MIF and is an established mediator of the deneddylation of CRL3, it serves as an attractive target to elucidate the mechanism by which MIF is impacting Nrf2 degradation. Understanding how MIF is impacting KEAP1 and CRL3 would provide important insight into MIF antioxidant-mediating capacity.

In conclusion, we have established that MIF is a necessary mediator of antioxidant responses in the context of CS in our *in vivo* and *in vitro* models. The loss of MIF increases the susceptibility to CS-induced injury through the increase of oxidative stress and the loss of the antioxidant responses. Furthermore we provide evidence for MIF regulating antioxidant responses by inhibiting proteasomal-mediated degradation of Nrf2. Antioxidant responses are important in the impeding the pathogenesis of COPD. These responses are altered in patients with COPD and are associated with increased oxidative burden and disease pathogenesis. In recognizing the importance of antioxidants in the context of increased oxidative burden during CS, our data provides insight for MIF's role in antagonizing CS-induced emphysematous remodeling. Finally, these results

demonstrate that MIF is a necessary mediator of antioxidant responses in vivo and a novel regulator of Nrf2 and it impacts its expression in proteasomal-dependent mechanism.

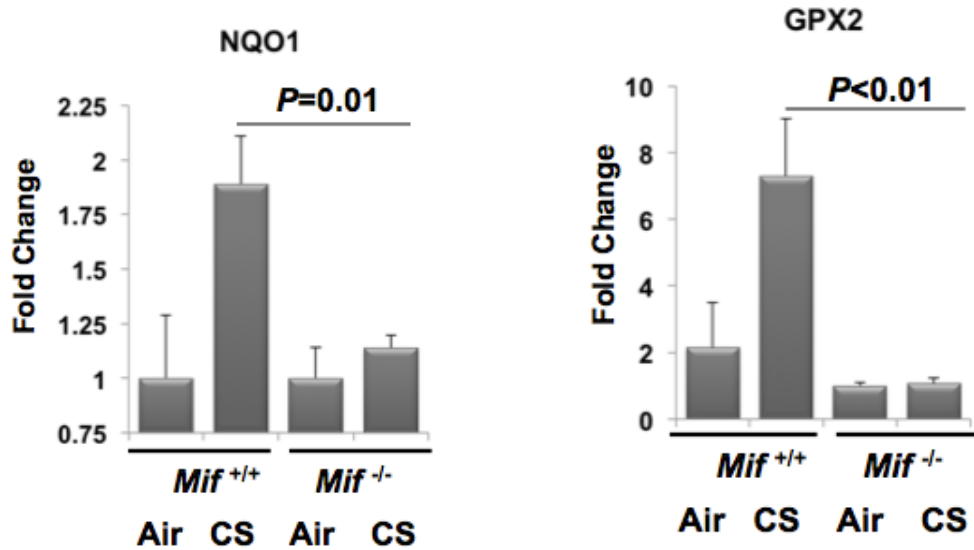


Figure 3-1: MIF is necessary for CS-induced antioxidant transcription in vivo.

Wildtype ($MIF^{+/+}$) and MIF knockout ($MIF^{-/-}$) that were exposed to either filtered air or CS for 3 days as previously described (Fallica, Varela et al. 2016) and total RNA was isolated from lung homogenates and analyzed by real time quantitative (RT-qPCR). Nrf2-dependent transcripts NQO-1 (A) and GPX2 (B) were quantified using Actin mRNA as a reference transcript. CS exposure induced significant changes in NQO1 and GPX2 mRNA in $MIF^{+/+}$ animals while there was a complete failure to increase Nrf2-dependent transcripts in the absence of MIF ($n=3/\text{group}$) ($p<0.01$).

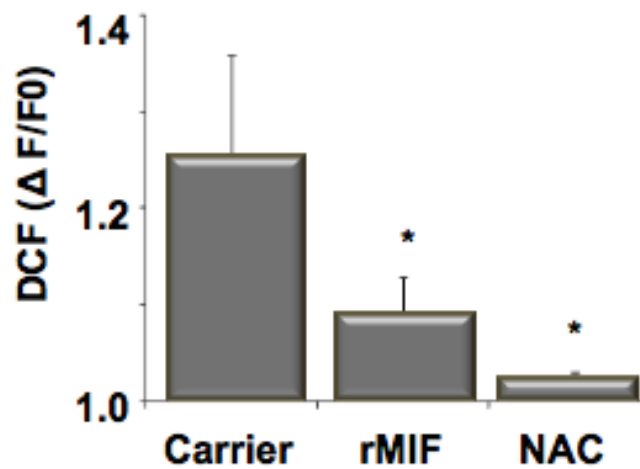


Figure 3-2: rMIF decreases CS-induced ROS accumulation in vitro. Primary HMVEC cells were loaded with the ROS-sensitive dye, DCFDA for 45 minutes. Cells were then pre-incubated with either carrier, rMIF(100ng/mL) or N-acetyl cysteine (NAC) for 4 hours prior to exposure to 20% CSE. Relative change in fluorescence from baseline (delta F/F0) was used to evaluate the CSE-induced ROS accumulation within the cells. Pre-treatment with rMIF significantly attenuated ROS production after CSE exposure (* $p < 0.01$).

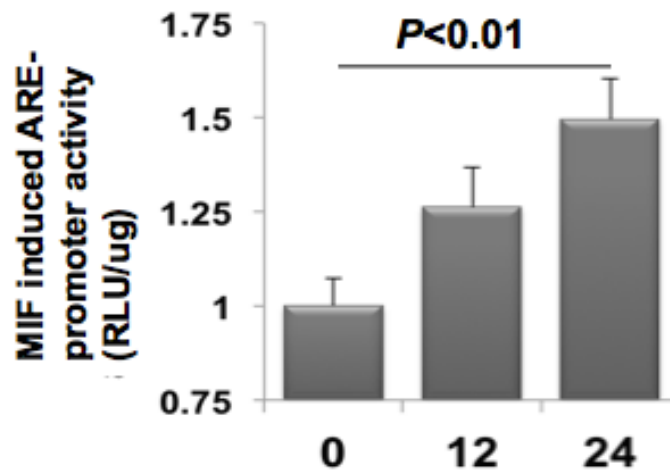


Figure 3-3: rMIF induces ARE-activity in vitro. HMVEC were transfected with a plasmid encoding the luciferase gene under the control of the ARE promoter and renilla gene constitutively expressed under the CMV promoter. Cells were then exposed to rMIF and lysed at 0, 12 and 24 hours. Luciferase activity was measured according to manufacturer's protocol, normalize to renillin and expressed as relative light units per microgram protein (RLU/ug). rMIF is sufficient to significantly increase ARE promoter activity in EC in vitro. Results based on 3 independent experiments (n=3/group) ($p < 0.01$).

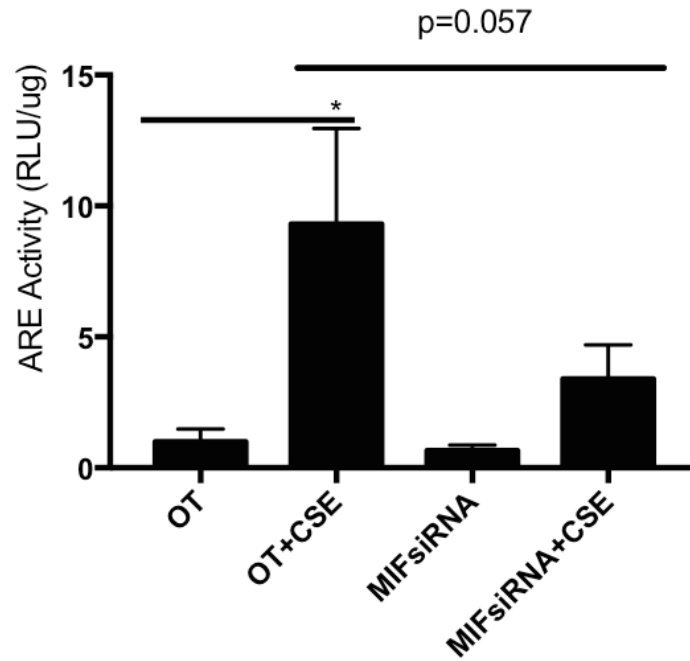
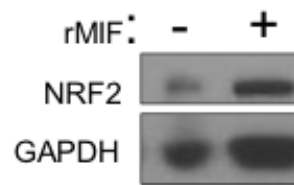
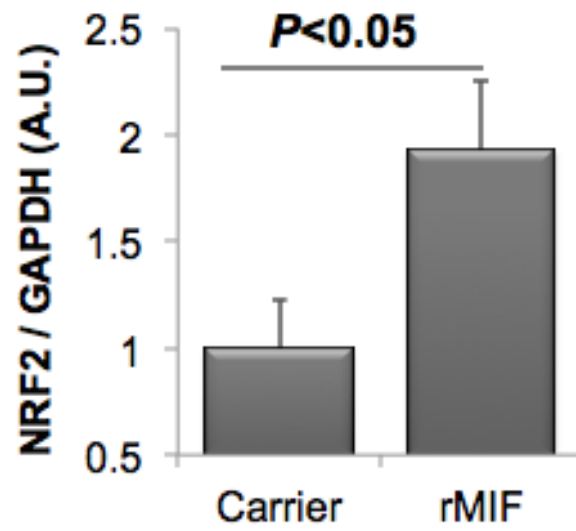


Figure 3-4: MIF loss attenuates CS-induced ARE activity. A. HMVEC_s were co-transfected with the ARE-luciferase reporter plasmid and either on-targeting control siRNA (OT) or MIF siRNA, conditions demonstrated to efficiently suppress MIF protein expression (data not shown). Cells were subsequently challenged with vehicle or 20% CSE for 24 hours and an assay to assess luciferase activity was completed according to manufacturers protocol (* $p < 0.05$).

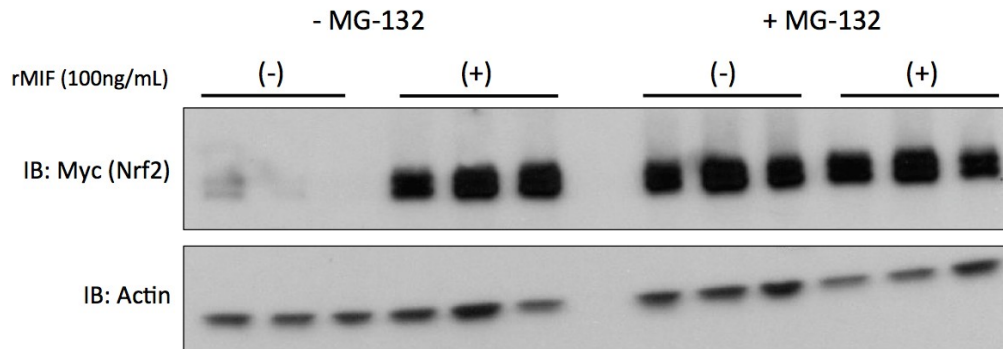
A.



B.



C.



D.

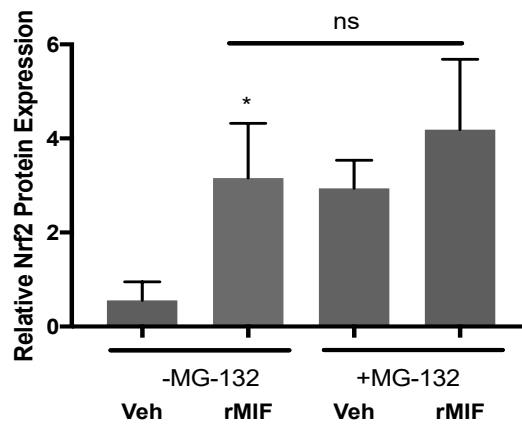


Figure 3-5: MIF-induced Nrf2 expression in ECs is proteasome-dependent. A. HMVEs were incubated with either carrier control or rMIF (100ng/mL). At 24 hours post-treatment, whole-cell lysates were prepared and were subject to western blot analysis as described in methods and materials and probed with anti-Nrf2 antibody and housekeeper, anti-GAPDH. Representative blot demonstrating increased Nrf2 protein in rMIF-treated EC. B. Quantification of the western by densitometric analysis ($p < 0.05$). C. rMIF increases expression of ectopic of Nrf2-myc in a proteasome-dependent manner. RMVECs were transfected with a myc-tagged Nrf2 plasmid and then treated with either vehicle control or rMIF (100ng/mL) for 24 hrs. They were then exposed to either DMSO or the proteasome-inhibitor, MG-132 (50 μ M) for 1 hr. Cells were then lysed and whole-cell lysates were prepared for western blot analysis and probed with for myc epitope actin.

rMIF induced a significant increase in Nrf2 protein. The ability to rMIF to further augment Nrf2 expression was lost in cells treated with MG-132 ($p=0.02$) (2-way ANOVA: NS).

Chapter 4

MIF Inhibits Proteasomal-Mediated Degradation of Nrf2 via CAND1 Induction

4.1 Abstract

The transcription factor, Nrf2 is the established master regulator of the antioxidant pathway. It is important in activating over 200 antioxidant genes that detoxify and neutralize ROS, thereby attenuating their detrimental biological effects (Itoh, Chiba et al. 1997). MIF is a proinflammatory cytokine that has many important biological functions, among which include the promotion of cell survival by inhibiting apoptosis (reviewed in (Bloom, Sun et al. 2016)). MIF deficient mice have increased ROS accumulation and emphysematous tissue remodeling in the lung after exposure to CS (Fallica, Boyer et al. 2014, Fallica, Varela et al. 2016). We have also previously shown that MIF may confer cytoprotection by positively regulating Nrf2 stability in a proteasomal-dependent mechanism (Chapter 3). Under basal conditions, Nrf2 is constantly targeted for proteasomal-mediated degradation by the KEAP1-CUL3 ligase complex (CRL3) (Villeneuve, Lau et al. 2010). KEAP1 serves as an adaptor molecule, connecting Nrf2 to the ubiquitination machinery, CRL3. CRL3 activity is regulated by the assembly and disassembly of its components, which are regulated by the cyclic addition and removal of the NEDD8 molecule, as well as the CRL negative regulator, CAND1. The process of neddylation/denndylation is catalyzed by two enzymes the neddyase, UBC12 and the deneddyase, JAB1 (Gong and Yeh 1999, Muromoto, Nakajima et al. 2013). Furthermore, MIF binds and negatively regulates JAB1 (Burger-Kentischer, Finkelmeier et al. 2005). Given MIF's ability to stabilize Nrf2 in a proteasomal-dependent mechanism and its negative regulatory effects on JAB1, we hypothesize that MIF is impacting Nrf2 stability via its effects on the KEAP1-CUL3 ligase complex.

Our results reveal that chronic CS exposure increased neddylation of CUL3 in the lung of wild type mice as compare to those exposed to air. This observation is lost in MIF deficient mice, implicating a MIF-dependent mechanism of CUL3 modification. Furthermore, MIF^{-/-} mice have increased JAB1 expression during CS exposure, which is coincident with decreased neddylation of CUL3, as well as depressed Nrf2-dependent responses and enhanced oxidant-mediated injury. Given that deneddylated CUL3 is associated with worse disease outcomes, we sought to test the hypothesis that driving the neddylation of CUL3 would lead to increase Nrf2 stability *in vitro*. We induced the neddylation of CUL3 *in vitro* by overexpressing the neddyase, UBC12. We found that increased neddylation was associated with an increase in Nrf2 protein expression and ARE activity. We also tested the hypothesis that MIF was a negative regulator of JAB1 in our model. MIF was not sufficient to impact JAB1 expression in EC *in vitro*, indicating that MIFs ability to modify ROS and Nrf2 expression *in vitro* are independent of direct effects on JAB1 expression. Finally, we found that exogenous MIF was sufficient to induce the expression of the negative regulator of CLR3, CAND1. CAND1 binds and inhibits CRL3 activity, thereby promoting the stabilization of the CRL's target substrate molecule. Overexpression of CAND1 in our model induced Nrf2 stability. Taken together, we demonstrate MIF's capacity to induce Nrf2 via its ability to upregulate the CAND1, the negative regulator of CRL3 activity.

4.2 Introduction

There is growing evidence demonstrating MIF's role in blunting ROS-induced injury, functioning in part to promote antioxidant responses *in vitro* and *in vivo*. Mathew et al showed that MIF is a necessary regulator of antioxidant gene expression in age-related susceptibility in radiation-induced lung injury. Aged MIF^{-/-} mice have enhanced susceptibility to oxidant-mediated lung injury, which was associated with significantly attenuated Nrf2 protein expression and its downstream antioxidant gene transcription (Mathew, Jacobson et al. 2013). We have previously demonstrated that MIF antagonizes CSE-induced ROS accumulation in EC in *in vitro* (Fig. 3-2). We have also found that MIF positively regulates the master regulator of the ARE, Nrf2, by interfering with its proteasome-mediated degradation (Fig. 3-5).

Nrf2 is a tightly regulated transcription factor that can be controlled both at the level of transcription and post-translation (Miao, Hu et al. 2005, Taguchi, Motohashi et al. 2011). It is important in the transcription of many genes involved in electrophilic stress, xenobiotic metabolism and glutathione synthesis (Okawa, Motohashi et al. 2006, Malhotra, Portales-Casamar et al. 2010). Under basal conditions, Nrf2 is rapidly turned over with a half-life of <20 minutes (Katoh, Iida et al. 2005). It is sequestered in the cytoplasm by KEAP1, an adaptor molecule and sensor of redox changes in the cell. KEAP1 assists in the polyubiquitination and degradation of Nrf2. Under conditions of oxidative stress however, KEAP1's reactive cysteine residues become oxidized, form disulfide bridges that induce its conformational changes and through mechanisms not yet fully elucidated, perturb its interaction with Nrf2 and thus stabilizes Nrf2 (Holland,

Hawkins et al. 2008). KEAP1 connects Nrf2 to the CRL3 (Kobayashi, Kang et al. 2004), the complex responsible for ligating ubiquitin to Nrf2 and targeting for degradation by 26S-mediated proteasome (Kirisako, Kamei et al. 2006).

This process generally occurs via three major classes of enzymes, ubiquitin activating enzymes (UAE or E1), ubiquitin-conjugating enzymes (E2) and finally, ubiquitin-protein ligases (E3) (Pickart 2001, Markson, Kiel et al. 2009). RING make up the largest group of E3 enzymes. RING E3 ligases bind a family of proteins known as cullin, which serve as molecular scaffolds by which other molecules can dock and form CRLs, the multisubunit complexes responsible for targeting a substrate molecule for ubiquitination (Hua and Vierstra 2011). CRLs differ in the types of cullins they form around and their constituents, but have they all have share the same general types of components. The basic components of the CRL consist of a scaffold (cullin), an adaptor molecule (i.e. KEAP1), E3 ligase, ubiquitin-loaded E2 ligase and a RING finger protein (RBX1 also known as ROC1). In eukaryotes, there are six different cullins (CUL1-5 and CUL7), which recognize different domains of adaptor molecules. CRL3, comprised of CUL3, specifically recognizes and interacts with the BTB/POZ domain (Genschik, Sumara et al. 2013), which is the characteristic domain found in KEAP1 (Furukawa, He et al. 2003, Geyer, Wee et al. 2003).

CRL activity is regulated by a number of ways chief among them is the cyclic addition and removal of a small ubiquitin-like molecule known as NEDD8. The reversible addition of the NEDD8 molecule to CRL leads to the activation of the complex and the recruitment of ubiquitin-loaded E2. Similar to ubiquitination, neddylation involves the mutli-step cascade consisting of activation (NEDD8 E1), conjugation

(NEDD8 E2 or UBC12) and ligation (NEDD8 E3) (Gong and Yeh 1999, Leidecker, Matic et al. 2012). NEDD8-UBC12 interacts with RBX1 to catalyze the transfer of NEDD8 from UBC12 to a lysine residue of the C-terminal domain of a Cullin molecule (Ohta, Michel et al. 1999). Neddylation is a reversible process, and NEDD8 can be removed by several different proteins with isopeptidase activities. JAB1, also known as CSN5, is a member of the COP9 signalosome that regulates neddylation by the enzymatic cleavage of NEDD8. It contains the MPN domain metalloenzyme (JAMM) motif, which confers the isopeptidase activity responsible for removing the NEDD8 molecule from cullin molecule (Schwechheimer and Deng 2001, Wee, Geyer et al. 2005).

Our previous study reveals that MIF is interfering with proteasomal-mediated degradation of Nrf2. We have thus used an *in vivo* model to specifically characterize the effects of MIF on CUL3. Our *in vivo* data reveals that CS exposure alters the neddylation status of CUL3. This is lost in the absence of MIF coincident with worse remodeling and enhanced expression of the deneddylase, JAB1. We then utilized RMVECs as our *in vitro* model to define the potential mechanism by which MIF impacts CUL3 neddylation status and Nrf2 stability. Previous *in vitro* studies indicate that MIF can modify the activity of the transcription factor AP-1, via direct binding and functional antagonism of JAB1. Kleeman and colleagues showed that MIF directly binds to JAB1 via residues 50-60, making Cys60 critical for its interaction with JAB1. Furthermore, they found that the addition of rMIF reversed the positive regulatory effects of JAB1 on AP-1 activating, it also antagonized enhanced JAB-1 mediated c-Jun amino terminal kinase (JNK) activity (Kleemann, Hausser et al. 2000). Given that MIF is an established negative regulator of JAB1 as well as our JAB1 findings *in vivo*, we sought to evaluate the effects of MIF on

JAB1 expression in ECs *in vitro*. Treatment with rMIF did not alter absolute JAB1 expression nor did it alter the neddylation status of CUL3 *in vitro*. This implicates another mechanism by which MIF is inducing Nrf2 stabilization in ECs *in vitro* (Kleemann, Hausser et al. 2000, Pyle, Korbonits et al. 2003). Given that deneddylation of CUL3 is associated with worse outcomes *in vivo* in MIF deficient mice, we postulated that promoting CUL3 neddylation in our system would be sufficient to stabilize Nrf2. We found that overexpression of the neddyase, UBC12 enhanced CUL3 neddylation and improved Nrf2 stability and ARE activity. Next we investigated MIF's effects on the negative regulator of the CRL, CAND1. Treatment of cells with rMIF was sufficient to upregulate CAND1 expression and forced overexpression of CAND1 induced Nrf2 expression. We have thus defined a novel mechanism by which MIF regulates Nrf2 degradation through its ability to positively regulate CAND1. This represents the first demonstration that CAND1 expression is responsive to any extracellular stimulus or circulating cytokine and provides the molecular framework to investigate aberrant CAND1 expression as a driver of disease severity in human emphysema/COPD.

4.3 Results

4.3.1 MIF Does Not Effect KEAP1 Expression *in vivo* and *in vitro*

We have previously shown that MIF is necessary for antioxidants responses *in vivo*. Furthermore, treatment of ECs with exogenous MIF significantly upregulated ARE activity as well as Nrf2 expression *in vitro* (Fig. 3-1, 3-3 & 3-4), suggesting the MIF is sufficient to drive antioxidant responses in pulmonary ECs. We have also demonstrated

MIF's capacity to stabilize Nrf2 via interference with its proteasome-mediated degradation. KEAP1 is the adaptor molecule that serves to bridge the CUL3-E2 ligase complex to Nrf2, to enable its poly-ubiquitination and subsequent degradation (Errington, Khan et al. 2012). KEAP1 structure responds to changes in cellular redox status, enabling it to unlatch from Nrf2, thereby promoting Nrf2 stabilization. Given KEAP1's key role in Nrf2 degradation and MIF's ability to promote its stability, we predicted that MIF antagonizes KEAP1 expression to promote its stability. We treated RMVECs with either vehicle control (0.1% BSA) or rMIF (100ng/ml). This dose was selected based on previous results that demonstrated its ability to protect ECs from CS-induced apoptosis (Damico, Simms et al. 2011) and sufficient to upregulate Nrf2 expression and ARE activity. Cells were lysed 24 hours post-transfection and KEAP1 expression was analyzed by western blot. We found that rMIF had no effects on KEAP1 expression *in vitro* (Fig 4-1).

Next, we investigated the effects of MIF on KEAP1 expression *in vivo*. Wild-type and MIF deficient mice were exposed to either air or CS acutely, for 3 days. CS exposure for this given time point has been proven to be sufficient to induce antioxidant responses in the lung and phenotypical differences are observed between wild type and MIF^{-/-} mice at this time point (Fallica, Varela et al. 2016). Total protein was isolated from whole lung homogenate. A western blot analysis of total KEAP1 protein expression revealed that MIF deficiency does not alter KEAP1 expression *in vivo*, indicating that MIF-induced Nrf2-stabilization is independent of alterations in KEAP1 expression (figure not shown).

4.3.2 Chronic CS Exposure Alters CUL3 Neddylation: Impact of MIF Deficiency

Given CUL3's critical role in the assembly of the CRL complex and in Nrf2 ubiquitination and subsequent degradation, we sought to investigate MIF's effects on the expression of CUL3 *in vivo*. MIF^{+/+} and MIF^{-/-} mice were exposed to either filtered air or CS chronically, for 6 months. Previous experiments have demonstrated that 6-month CS exposure is sufficient to induce enhanced emphysematous tissue remodeling in MIF deficient mice (Fallica, Boyer et al. 2014). Protein was isolated from whole lung tissue that was homogenized in cell lysis buffer. Expression of CUL3 was then analyzed by western blot. There were no observable differences in absolute CUL3 expression in lung homogenates from MIF^{+/+} and MIF^{-/-} mice exposed at baseline (i.e. air) or following CS.

Recognizing that we did not observe MIF-dependent differences in KEAP1-CUL3 protein, we investigated the effects of MIF on the well-characterized post-translational modification (PTM) of CUL3, specifically its neddylation status. Given the important role of NEDD8 in regulating CRL activity (Kamitani, Kito et al. 1997, Hori, Osaka et al. 1999) and MIF's ability to regulate antioxidants, we investigated the role of MIF on CUL3 neddylation. We found no difference in the neddylation of CUL3 in MIF^{+/+} and MIF^{-/-} at baseline in mice exposed to filtered air. Thus, MIF does not seem to be necessary in CUL3 neddylation under basal conditions. Following exposure of MIF^{+/+} mice to prolonged CS, we observed a significantly increase in the neddylation of CUL3 by about 1.5-fold ($p < 0.01$) (Fig. 4-2). Interestingly, we found that CS-induced neddylation of CUL3 is completely lost in MIF^{-/-} mice and resembles the neddylation status of mice exposed to air (Fig. 4-2), implicating MIF's role in mediating CUL3 activity under conditions of increased oxidative burden.

4.3.3 Chronic CS Exposure Induces Expression of the Deneddylase, JAB1, in the Absence of MIF

In recognizing that CS increases the CUL3 neddylation, an observation that is lost in MIF^{-/-} mice, we sought to determine if this was associated with altered expression of the deneddylating enzyme, JAB1 *in vivo*. MIF is an established negative regulator of JAB1 function. To test the effects of MIF on JAB1 *in vivo*, we investigated the JAB1 expression in total lung homogenates of MIF^{+/+} and MIF^{-/-} mice that were chronically exposed to either filtered air or CS for 6 months. We would predict that the differences observed in CUL3-neddylation observed in MIF^{+/+} and MIF^{-/-} mice could be linked to altered JAB1 expression and/or activity. Western blot results reveal that MIF deficiency does not alter JAB1 expression under basal conditions (Fig. 4-3). CS exposure itself is not sufficient to affect JAB1 expression in MIF^{+/+} mice. In contrast, JAB1 expression is significantly upregulated by about 4-fold in MIF deficient mice relative to their MIF^{+/+} counterparts exposed to chronic CS ($p<0.01$) (Fig. 4-3). Thus, we observe a differential effect of CS on CUL3 neddylation status and expression of the deneddylase, JAB1, as a function of MIF expression in the intact murine lung.

4.3.4 Exogenous MIF Does Not Alter Expression of Total Cul3 or NEDD8-CUL3 *in vitro*

Exogenous MIF has been demonstrated to protect CS-induced EC apoptosis (Damico, Simms et al. 2011), attenuate ROS accumulation in cells exposed to CSE (Chapter 3, Fig. 3-2), upregulate Nrf2 expression and ARE activity in ECs (Fig. 3-3 & Fig. 3-4). Furthermore, MIF^{-/-} mice display greater susceptibility to the CS-induced

emphysematous remodeling, which is associated with decreased CUL3 neddylation. Taken together, we aimed to investigate the effects of exogenous MIF on CUL3 neddylation status in EC *in vitro*. We postulated that treatment of rMIF would increase the neddylation of CUL3, leading to enhanced Nrf2 expression.

RMVECs were exposed to either vehicle (0.1% BSA in PBS) or rMIF (100ng/mL) for 24 hours. This time point has been previously established for significant induction of ARE activity and Nrf2 expression (Chapter 3). Cells were then lysed and total CUL3 expression in whole cell lysates was analyzed by western blot. We have found that rMIF was not sufficient to alter total CUL3 expression. Furthermore, there were no differences in neddylated CUL3 (NEDD8-CUL3) (Fig. 4-4), implicating MIF's role in mediating Nrf2 expression *in vitro* are independent of changes in CUL3 neddylation status.

4.3.5 Overexpression of the Neddyase, UBC12 Increases NEDD8-CUL3

Given our *in vivo* findings, we postulated that enhanced CUL3 neddylation would be associated with increased antioxidant responses. We therefore attempted to drive the neddylation of CUL3 by forced expression of the neddyase, UBC12. We predicted that overexpression of UBC12 would increase CUL3 neddylation. We transfected RMVECs with either pcDNA or hemagglutinin (HA)-tagged UBC12 plasmid under the CMV promoter. Western blotting of the HA-tag confirmed expression of the transgene. As we predicted, overexpressing a UBC12 plasmid was sufficient to drive the neddylation status of CUL3 by approximately, 1.5-fold ($p=0.02$) (Fig. 4-8).

4.3.6 UBC12 Overexpression Induces Nrf2 Expression *in vitro*

Chronic CS exposure is associated with enhanced CUL3 neddylation and MIF loss results in decreased CUL3 neddylation, absent Nrf2-dependent gene expression, increased ROS, increase cytotoxicity and enhanced emphysematous remodeling. In order to demonstrate the effects of UBC12-induced CUL3 neddylation on EC antioxidant capacity, we co-transfected RMVECs with either pcDNA or UBC12-HA and Nrf2-myc plasmids and harvested cells within 24 hours of transfection. Driving the expression of UBC12 in our system significantly upregulate CUL3 neddylation (Fig. 4-8), which was also associated with a nearly 2-fold increase in Nrf2 expression ($p<0.01$) (Fig.4-9A, 4-9B). Next, we investigated the effects of enhanced CUL3 neddylation on ARE activity. Either pcDNA or UBC12-HA and a luciferase construct under the control of the ARE (as described in Chapter 3) were transfected in RMVECs. Cells were harvested within 24 hours of transfection and assayed the lysates according to manufacturer's protocol. Our results indicate that UBC12 overexpression significantly up-regulates ARE activity by over 2-fold ($p=0.021$) (Fig. 4-9C). Thus, CUL3 neddylation is sufficient to stabilize Nrf2/ARE activity in EC.

4.3.7 JAB1 Does Not Affect the Neddylation of CUL3 *in vitro*

Our *in vivo* data revealed that MIF loss during CS exposure was associated with an increase in JAB1 expression, which was coincident with decreased CUL3 neddylation. These observations were also associated with worse emphysematous remodeling (Fallica, Boyer et al. 2014). Previous work from our lab suggests that JAB1 antagonizes Nrf2 expression, as JAB1 loss led to enhanced Nrf2 expression and ARE activity (data not

shown). In order to elucidate the effects of CUL3 neddylation on Nrf2 stabilization, we attempted to induce the deneddylation *in vitro* by the overexpression of JAB1. We transfected RMVECs with either an empty pcDNA vector or a myc-tagged JAB1 plasmid under the control of the CMV promoter. Cells were then harvested within 24 hours post-transfection, to ensure expression of the tagged transgene. Whole cell lysates were then run on an SDS-PAGE gel for western blot analysis of neddylated-CUL3 expression.

We expected that overexpressing the deneddylase, JAB1 would promote CUL3 deneddylation. Our results however, show that JAB1 overexpression was not sufficient to drive the deneddylation of CUL3 *in vitro*, as there were no observable differences in CUL3 neddylation in cells transfected with pcDNA as compared to those transfected with the JAB1 overexpressing plasmid (Fig. 4-5). We therefore attempted use an alternative approach to address the role of JAB1 in CUL3 neddylation *in vitro*. HMVECs were transfected with either OT or siRNA targeted against JAB1. We then harvested cells 48 hours post-transfection, to allow for sufficient JAB1 knockdown. Protein was isolated from whole cells and lysates were run on an SDS-PAGE gel for western blot analysis. We predicted that the abrogation of JAB1 would lead to an increase in the neddylation of CUL3. Our results show that while we successfully knocked down JAB1 by about 70% ($p<0.01$) (Fig. 4-6B), it did not affect total or neddylated CUL3 expression, suggesting JAB1 does not significantly impact CUL3 neddylation status *in vitro* (Fig.4-6C & 4-6D).

4.3.8 Enhanced Expression of Dominant Negative UBC12 Does not Alter CUL3 Neddylation *in vitro*

In a parallel experiment, we attempted to overcome challenges of deneddylation CUL3 by JAB1 overexpression by using an alternative approach and inhibiting UBC12 directly via expression of a dominant negative form of the enzyme (dnUBC12). The dnUBC12 construct has a specific point mutation (C111S) in the catalytic site of the enzyme, inhibiting its function (Wada, Yeh et al. 2000). We therefore transfected RMVECs with either pcDNA with an empty vector or plasmid encoding dnUBC12 with the hemagglutinin epitope tag (HA) and used western blotting to analyze the effects on CUL3 neddylation. Total cell lysates were collected 24 hours post transfection. Transgene expression was confirmed with Anti-HA antibodies. We predicted that overexpressing dnUBC12, that it would drive the deneddylation of CUL3. Our results however, reveal, that while we expressed the dnUBC12-HA plasmid, it did not affect the neddylation status of CUL3 *in vitro* (Fig. 4-7).

4.3.9 MIF Does Not Alter JAB1 Expression *in vitro*

Prior studies suggest that JAB1 suppresses Nrf2, as demonstrated by increased Nrf2 in JAB1 deficient EC *in vitro* (Fallica Dissertation, 2015). Our data here indicate that MIF is sufficient to stabilize Nrf2 suggesting that it may modulate Nrf2 via effects on JAB1 expression. In order to test this hypothesis this, we utilized gain of and loss of function approaches. HMVECs were transfected with either OT control or MIF siRNA. We expected that the loss of MIF would drive JAB1 expression. MIF loss *in vitro* however did not alter JAB1 expression *in vitro* (data not shown), suggesting that MIF

does not negatively regulate JAB1 expression in ECs under basal conditions. We then asked if treatment with rMIF would down-regulate JAB1 expression. In order to test this, HMVECs were treated with either vehicle control or rMIF (100ng/mL) for 24 hours and then lysed. Western blotting was used to evaluate JAB1 expression in whole cell lysate. We found that treatment with rMIF was not sufficient to alter JAB1 expression (Fig. 4-10).

4.3.10 Exogenous MIF Increases the Expression of the CRL Inhibitor, CAND1

Activity of the CRL is not only dependent on the neddylation status, but also the assembly and disassembly of the complex, which is regulated by CAND1. CAND1 binds to and inactivates the CRL, preventing the formation of the active CRL-E3 ligase complex. We therefore, wanted to test the hypothesis that rMIF is negatively impacting CUL3 via effects on the negative regulator, CAND1. RMVECs were treated with either vehicle control (0.1% BSA) or rMIF (100ng/mL) for 24 hours. We harvested cells and used immunoblotting to analyze MIF's effects on CAND1 expression. We found that rMIF significantly induced CAND1 expression by about 50% ($p=0.02$) (Fig. 4-11A, 4-11B). We also show that rMIF is sufficient to alter the ratio of CAND1 to CUL3 by about 1.5-fold ($p=0.03$) (Fig.4-11C). We therefore provide evidence for MIF as a novel positive regulator of an established CRL inhibitor, CAND1.

4.3.11 CAND1 Overexpression Increases Nrf2 Protein

Given CAND1's role in negatively regulating of the CUL3 ligase complex, we predicted that overexpressing CAND1 would be sufficient to drive Nrf2 protein

expression in our model. To test this we co-transfected RMVECs with myc-tagged Nrf2 and either the empty vector, pcDNA or a myc-tagged CAND1 plasmid under the control of the CMV promoter. Ectopic CAND1 and Nrf2 have different mobility's allowing for their identification based on molecular weight. Cells were then harvested within 24 hours of transfection. Ectopic Nrf2 and CAND1 expression in whole cell lysates were analyzed by western blot. We observed that overexpression of CAND1 was sufficient to drive Nrf2 expression by nearly 2-fold ($p=0.015$) (Fig.4-12), potentially via its capacity to inhibit activation of CRL3.

4.4 Discussion & Conclusion

The Nrf2-antioxidant pathway is critical in mitigating the effects of oxidative stress. It provides the first line of defense against ROS. Once activated, Nrf2 binds to the ARE, the DNA element found in the promoter region of over 200 antioxidant genes. Its importance in CS-induced lung injury was demonstrated in Nrf2 deficient mice. These mice had increased markers of oxidative stress, DNA damage, attenuated antioxidant responses and enhanced apoptosis (Iizuka, Ishii et al. 2005). Furthermore, chronic CS exposure significantly blunts Nrf2-expression, which is associated with increased inflammation (Garbin, Fratta Pasini et al. 2009). Nrf2 is a dynamically regulated molecule and under basal conditions and is constitutively generated and targeted for degradation by the KEAP1-CUL3 ligase complex. This allows for rapid changes in protein expression in responses to environmental stimuli (Chan and Kan 1999, Itoh, Ishii et al. 1999). We have previously shown that MIF is a necessary mediator of antioxidant

transcriptional responses during acute CS exposure *in vivo*. It also up-regulates Nrf2 expression in a proteasomal-dependent manner and ARE activity *in vitro* (Chapter 3). In this study, we have identified MIF as a novel positive regulator of CAND1, an inhibitor of the CRL machinery that is required to ubiquitinate and target Nrf2 for degradation (Zheng, Yang et al. 2002). Further, we provide evidence that CAND1 expression can stabilize Nrf2 expression.

Having established MIF as positive regulator of Nrf2, we sought out to determine if MIF altered key Nrf2's regulatory components, KEAP1 and CUL3. This was accomplished in both primary cells *in vitro* and in the intact murine lung. We manipulated its expression by utilizing gain and loss-of-function approaches via exogenous rMIF treatment and siRNA targeted against MIF *in vitro* and knockout mice *in vivo*. We first investigated MIF's effects on KEAP1, the adaptor molecule that serves to link Nrf2 to the CUL3 ligase complex. Given KEAP1's role in suppressing Nrf2, we predicted that exogenous MIF would inhibit KEAP1 expression *in vitro*. We have found that treatment of cells with rMIF failed to suppress KEAP1 expression (Fig. 4-1). Our *in vivo* data reveals that KEAP1 expression is unaffected by the absence of MIF (data not shown). Similarly, we tested the effects of rMIF on the scaffold, CUL3 *in vitro*. If MIF functioned via direct effects on CUL3, we would expect that rMIF should alter CUL3 expression, but we found that exogenous MIF had no effects on absolute CUL3 expression in the time point we were investigating (Fig. 4-4). In summary, we have no evidence to suggest that MIF is suppressing the expression of key CRL3 components, implicating mediating an alternative mechanism.

In recognizing that MIF's capacity to positively regulate Nrf2 is independent of changes in absolute expression of KEAP1 and CUL3, we directed our efforts to identifying MIF's effects on established regulators of the CRL complex activity. One such mechanism of CRL regulation is via PTM specifically, neddylation (Lyapina, Cope et al. 2001, Cope, Suh et al. 2002). We first addressed if MIF deficiency alters the neddylation of CUL3 in our *in vivo* CS-induced emphysema model. Our data reveals that CS promotes the neddylation of CUL3 in MIF^{+/+} mice, which was not observed in MIF^{-/-} mice. This implicates MIF as a positive regulator of neddylation in the context of oxidative stress (Fig. 4-3). CS-induced neddylated CUL3 in MIF^{+/+} mice have increased antioxidant capacity and decreased emphysematous remodeling. We therefore, aimed to test the hypothesis that driving CUL3 neddylation would be associated with increased Nrf2 and antioxidant capacity. Overexpression of the neddyase, UBC12, increased CUL3 neddylation *in vitro*. UBC12-mediated CUL3 neddylation was also associated with increased Nrf2 expression and ARE activity by nearly 2-fold (Fig. 4-9). Taken together, these results suggest that increasing the neddylation of CUL3 is associated with increased antioxidant capacity.

Interestingly, our *in vivo* data suggests a potential role for JAB1 in the differential CUL3 neddylation status observed in wild type and MIF deficient mice. Changes in CUL3 occurred in the presence of enhanced expression of the established deneddylase and MIF binding partner, JAB1. This led us to speculate that MIF's ability to positively regulate Nrf2 is via JAB1 suppression and promotion of CUL3 neddylation. In order to test this hypothesis, we used two complementary approaches to test JAB1's ability to alter CUL3 neddylation. In the first approach, we overexpressed JAB1 in RMVECs to

test if it was sufficient to promote CUL3 deneddylation. We observed no differences in NEDD8-CUL3 in JAB1 overexpressing cells (Fig. 4-5) indicating that either the kinetics of the deneddylase reaction was either insufficient to observe measurable differences during the 24 hour reaction or that the transfection efficiency was insufficient to see measurable differences in the entire population. Next, we knocked down JAB1 expression in HMVECs using siRNA. This second technique is highly efficient and prior studies demonstrate that greater than 95% of the monolayer of cells are transduced duplex RNA (Damico, Chesley et al. 2008). We predicted that by knocking down the deneddylase we would observe an increase in the neddylation of CUL3. We found no differences in CUL3 neddylation in JAB1 deficient cells. Published works suggest that changes in the neddylation status of CUL3 can also alter absolute CUL3 expression via alterations in auto-ubiquitination and subsequent degradation (Duda, Borg et al. 2008, Fischer, Scrima et al. 2011). Despite these reports, we found that manipulation of the deneddylase (directly via JAB1 or indirectly via dnUBC12) did not alter absolute CUL3 (Fig. 4-6 & Fig. 4-2). In summary, these results suggest that despite the observed increase JAB1 in MIF^{-/-} animals exposed to smoke, JAB1 is not responsible for the ability of MIF to positively regulate Nrf2 *in vitro*.

In a third approach to alter CUL3 neddylation, we attempted to inhibit the function of UBC12 by overexpressing a dominant negative form of the protein (dnUBC12) with an impaired catalytic site (Wada, Yeh et al. 2000). Our results revealed that the overexpression of dnUBC12 was not sufficient to drive alter CUL3 neddylation status or alter absolute CUL3 expression (Fig. 4-7). This failure may reflect our inability

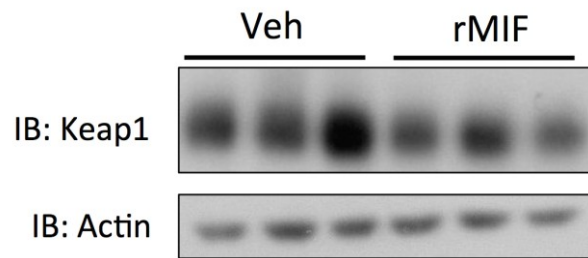
to transfect the transgene into the majority of cells in the monolayer or be a function of kinetics.

In addition to investigating the role of CUL3 PTM, we studied MIF's roles on another regulator of CUL3 activity, CAND1. Inactivation of cullins is a two-step process. First, it requires the activity of JAB1 to remove the NEDD8 molecule and then it requires the function of the CRL inhibitor (CAND1). CAND1 is a 120-kDa protein that exclusively recognizes deneddylated cullins. After the enzymatic removal of NEDD8 from the CRL by JAB1, CAND1 is then able to bind both CUL and RING finger protein (Yogosawa, Makino et al. 1996, Kawakami, Chiba et al. 2001, Boutten, Goven et al. 2011). We have found rMIF increased CAND1 protein expression *in vitro* by about 50% (Fig. 4-11). Further, the forced overexpression of CAND1 in RMVECs was sufficient to increase Nrf2 expression by over 2-folds (Fig. 4-12). Work from Lo and Hannick 2006 demonstrates that CAND1 can compete with KEAP 1 for CUL3 binding, thus antagonizing Nrf2 degradation. In cells made deficient of CAND1, KEAP1 preferentially binds CUL3 increasing KEAP1 ubiquitination and degradation. Thus, CAND1 appear to dynamically regulate Nrf2 with increased expression stabilizing via direct binding to CUL3 and absolute loss decreasing degradation indirectly by required via direct effects on KEAP (Lo and Hannink 2006). CAND1's role in cellular responses to oxidative stress has not been fully elucidated. Taken together, these data demonstrate CAND1's importance in regulating efficient CRL3 activity (Lo and Hannink 2006, Bosu and Kipreos 2008).

The limited characterization of CAND1 function in the context of oxidative injury serves to highlight the novelty of our observation, as MIF-induced CAND1 expression

provides a potential mechanism by which Nrf2 is modified in the setting of oxidative stress. Additional studies should be directed at determining the effects of ROS on CAND1 expression and function, as well as the identifying effects of CAND1 in potentially mitigating CS-induced apoptosis in ECs. It is unclear if MIF's ability to induce CAND1 expression is unique to pulmonary ECs, or more it is a more generalizable finding in other cell types and species. Future studies may also be designed to identify the effects of MIF deficiency on CAND1 expression both *in vivo* and *in vitro*. Furthermore, the interaction between MIF and CAND1 can further be elucidated with rescue experiments. We have previously demonstrated that MIF deficiency enhances CS-induced apoptosis (Damico, Simms et al. 2011). Studies can be designed aim to elucidate the effects of CAND1 overexpression in the context of CSE-induced apoptosis in MIF deficient cells. While more work is required to further characterize the effects of CAND1 in our EC model, we provide evidence that MIF is impacting Nrf2 stability in a proteasomal-dependent mechanism via its capacity to regulate the CRL-inhibitor, CAND1. Antioxidant responses are altered in the context of CS-induced emphysema and identifying CAND1's function in our *in vitro* and *in vivo* models provides insight into disease pathogenesis and potential therapeutic targets.

A.



B.

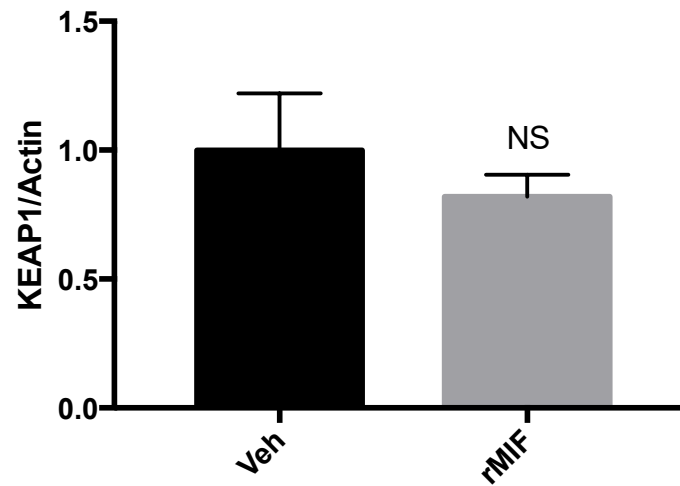


Figure 4-1. rMIF does not effect KEAP1 expression *in vitro*. Western blot of RMVECs that were treated with either vehicle (0.10% BSA in PBS) or rMIF (100ng/mL), protein was isolated 24 hours post-treatment. Immunoblot A.) KEAP1 and Actin B.) Densitometric analysis of western blot ($p=0.256$).

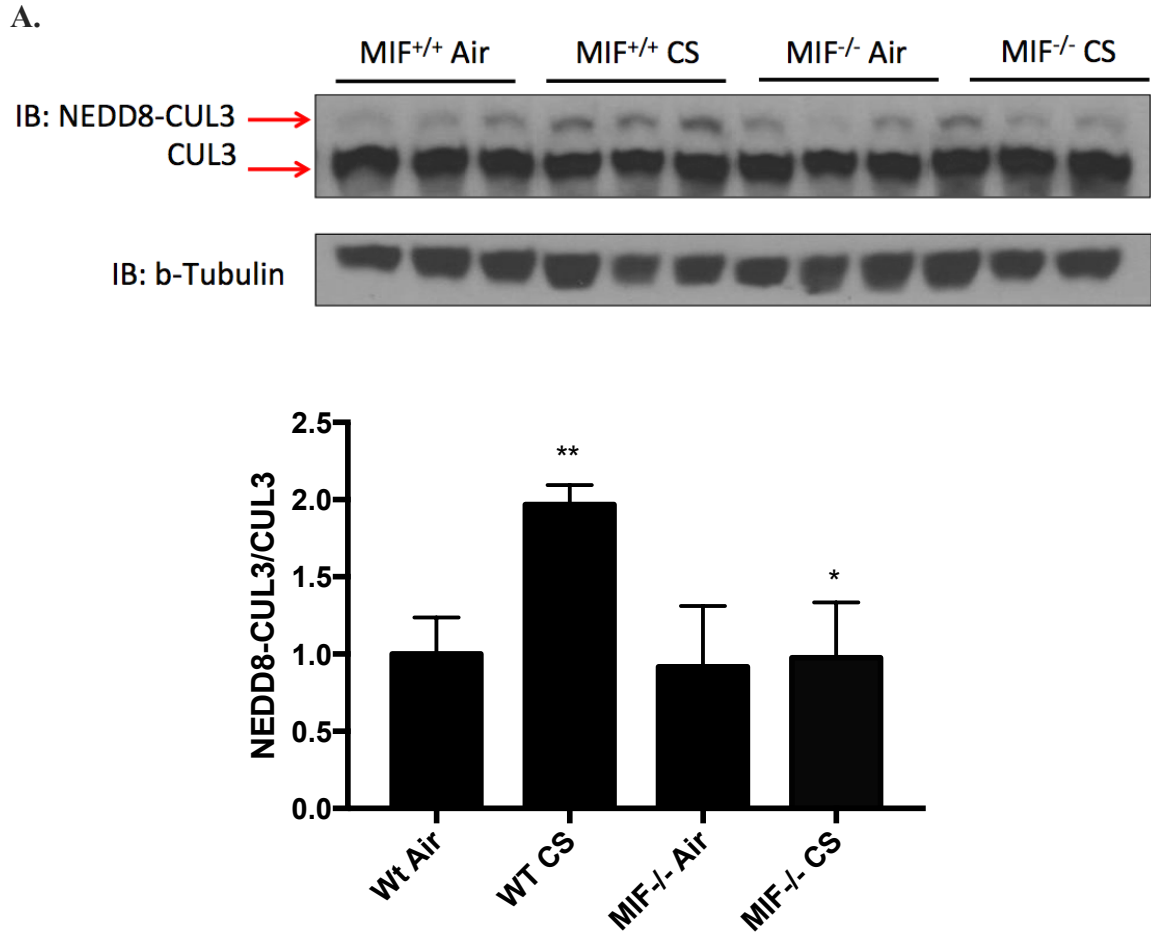


Figure 4-2: MIF-dependent CUL3 neddylation *in vivo*. Western blot analysis of whole-lung homogenate of MIF^{+/+} or MIF^{-/-} mice exposed chronically for 6 months to either filtered air or CS. A. Immunoblot of Cul3 and neddylated Cul3. B. Graphical representation of densitometric quantification of the neddylation fraction over the deneddylated fraction (n=3/group) (**p<0.01; *p=0.01) (2-way ANOVA p<0.05).

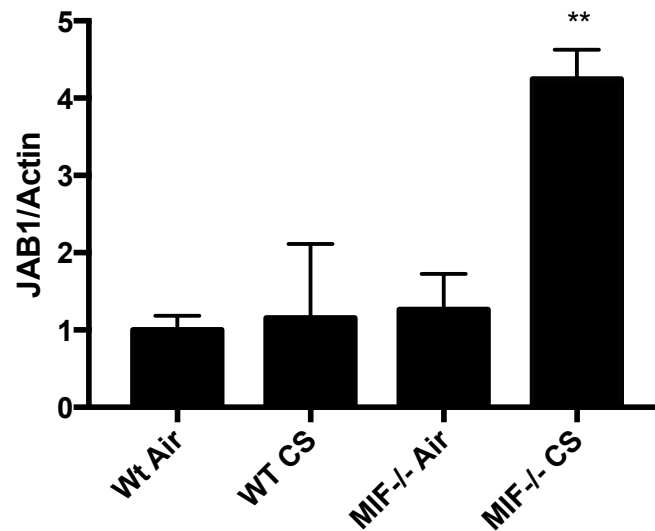
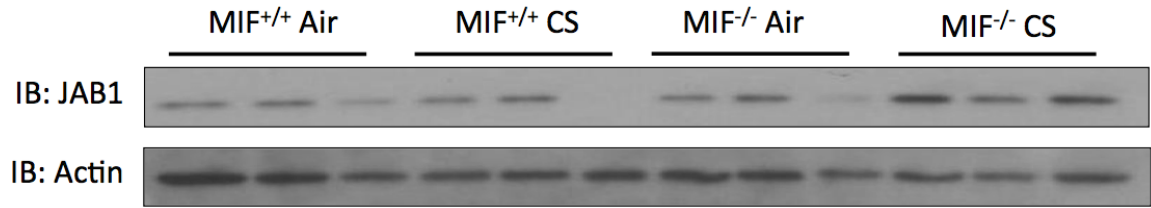
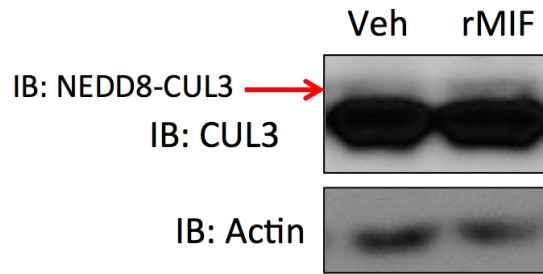


Figure 4-3: MIF-deficiency increases CS-induced JAB1 expression in vivo. MIF^{+/+} or MIF^{-/-} mice exposed chronically for 6 months to either air or CS A. Western blot analysis of JAB1 expression in the lungs of MIF^{+/+} and MIF^{-/-} mice exposed to either air or CS. Lungs were isolated, homogenized (see methods and materials) and whole lung homogenate was immunoblotted was used to analyze expressions of JAB1 and Actin (** $p < 0.01$). B. Quantification of the densitometric analysis of western blot (n=3/group) (2-way ANOVA $p < 0.05$).

A.



B.

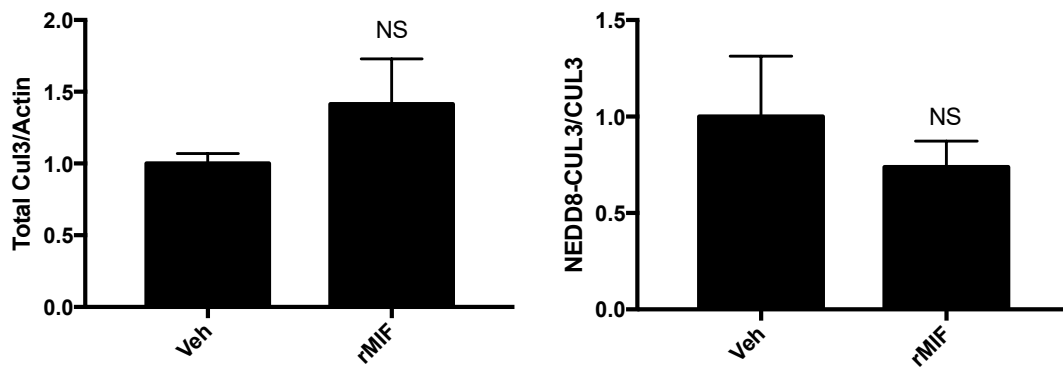
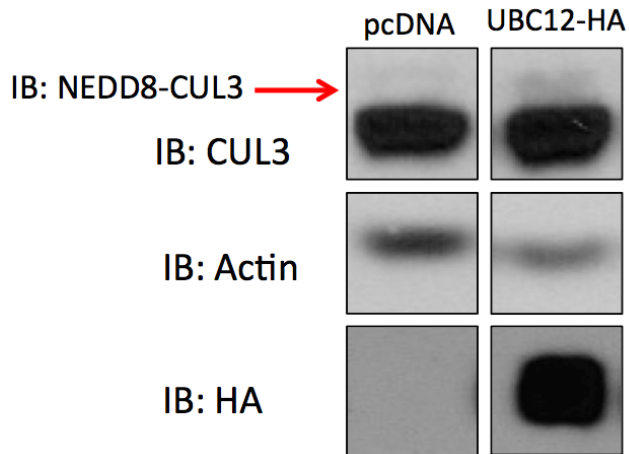


Figure 4-4: rMIF does not alter CUL3 neddylation *in vitro*. RMVECs were treated with either vehicle (0.10% BSA in PBS) or rMIF (100ng/mL) and protein was isolated 24 hours post-treatment. Western blot analysis of whole-cell lysates and analyzed for A.) CUL3, NEDD8-CUL3 and Actin expression B.) Densitometric analysis of total CUL3 and NEDD8-CUL3.

A.



B.

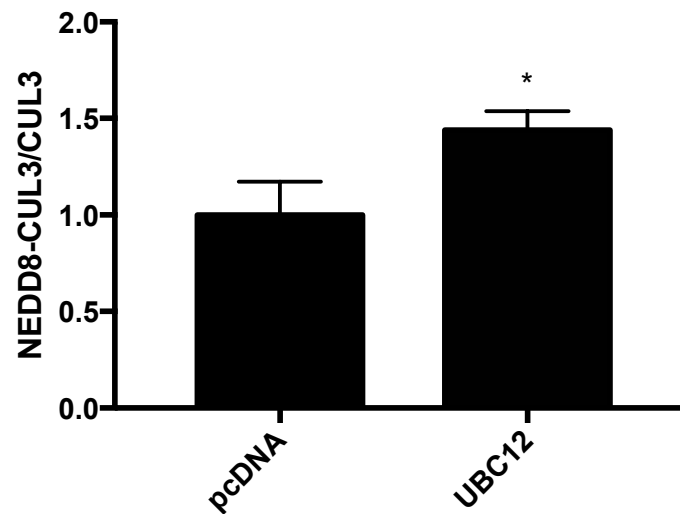
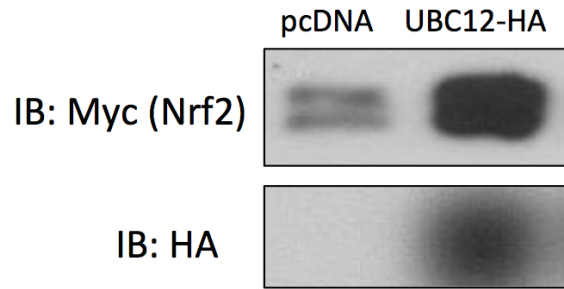
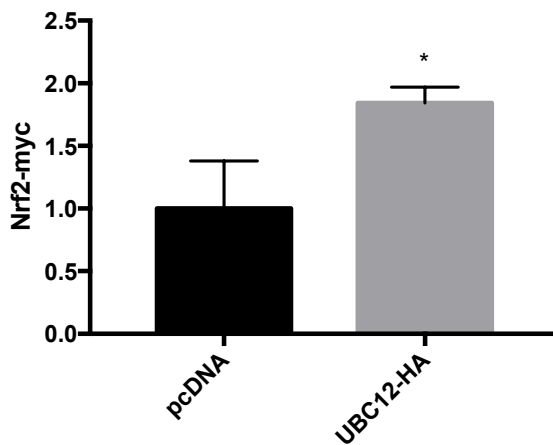


Figure 4-5: UBC12-mediated CUL3 neddylation. RMVECs were transfected with 1 μ g of either pcDNA or UBC12-HA and then lysed about 24 hours post-transfection. Whole-cell lysates were run on an SDS-PAGE gel Western blot analysis of A.) NEDD8-CUL3, actin and ectopic HA-tag B. Densitometric analysis of NEDD8-CUL3 (* $p=0.018$).

A.



B.



C.

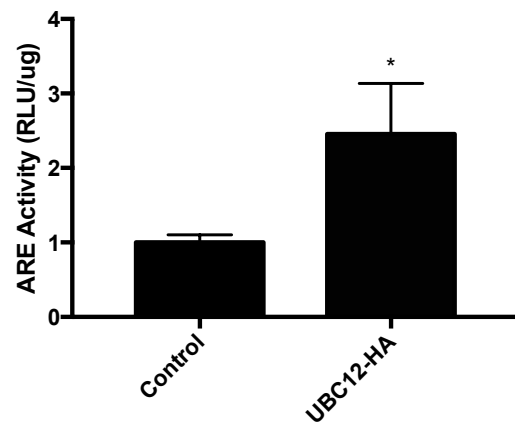


Figure 4-6: UBC12-driven Nrf2 protein expression and ARE activity. RMVECs were co-transfected with 0.5ug of Nrf2-myc and 0.5ug of either pcDNA or UBC12-HA, cells were harvested about 24 hours post-transfection. Western blot analysis of A.) myc-tagged Nrf2 and HA-tag B.) Graphical representation of immunoblot densitometry of Nrf2-myc (** $p < 0.01$) C.) Luciferase activity of cells were co-transfected with a 0.3ug of ARE-luciferase construct and 0.70ug of either pcDNA or UBC12-HA, then harvested within 24 hours of transfection. Luciferase assay was completed according to manufacturer's protocol (* $p = 0.021$).

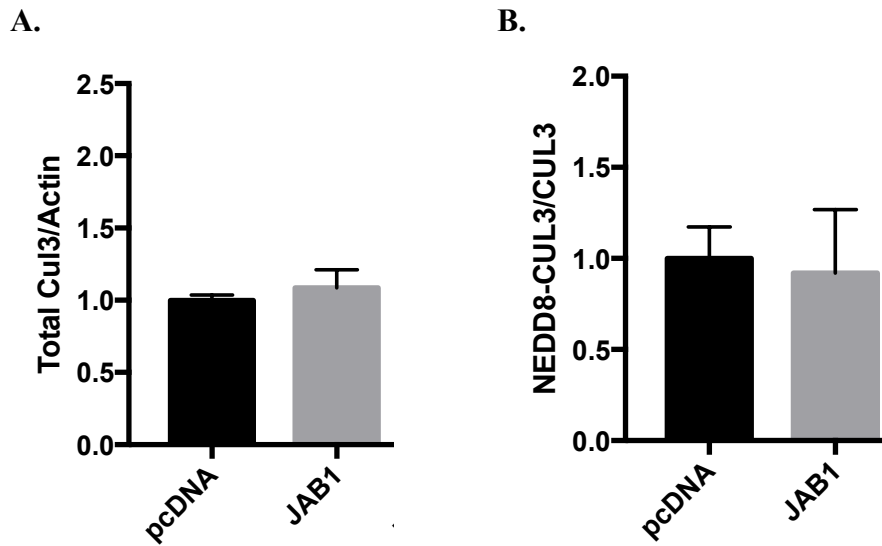
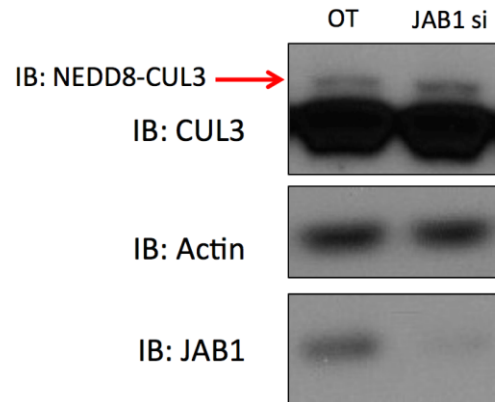


Figure 4-7: JAB1 overexpression does not alter CUL3 neddylation pulmonary ECs.

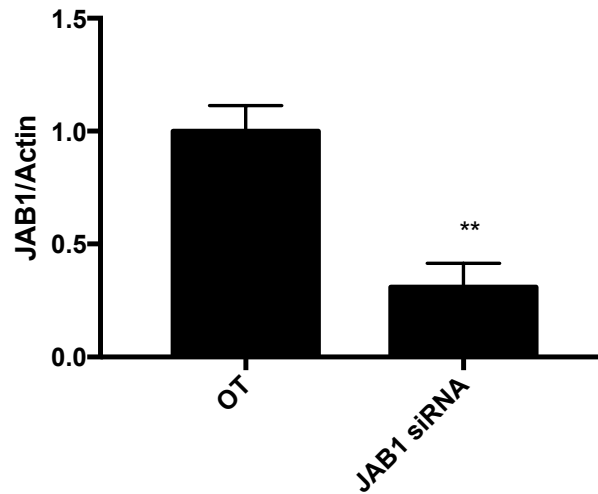
RMVECs were transfected with 1 μ g of either pcDNA or myc-tagged JAB1 plasmid in 6-well plates, cells were then harvested 20-24 hours post-transfection and whole cell lysates were then analyzed by immunoblotting with anti CUL3 and actin antibodies. Densitometric analysis of total

A.) CUL3 B.) NEDD8-CUL3.

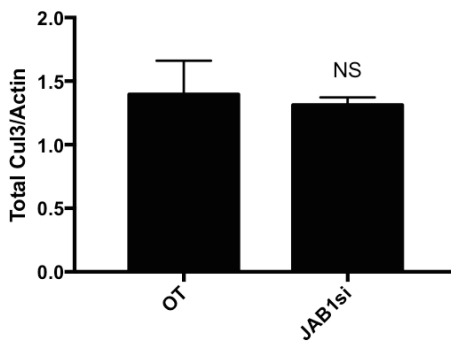
A.



B.



C.



D.

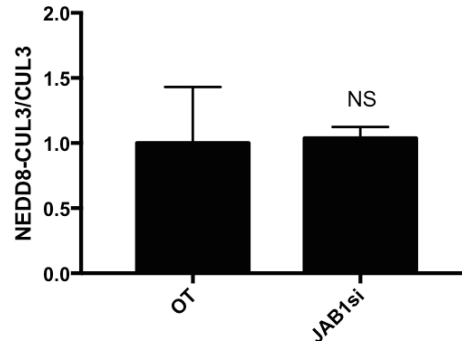
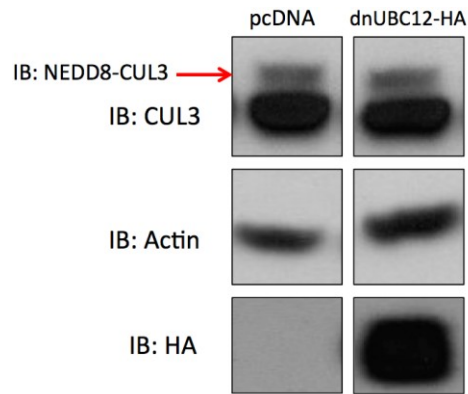


Figure 4-8: JAB1 deficiency does not alter the neddylation of CUL3. HMVECs were transfected with either OT or JAB1 siRNA and were then harvested 48 hours post-transfection and whole-cell lysates were then analyzed by immunoblotting with A.) anti-CUL3, anti-Actin and anti-JAB1 antibodies. Densitometric analysis of B.) JAB1 ($p < 0.01$) C.) total CUL3 and D.) NEDD8-CUL3.

A.



B.

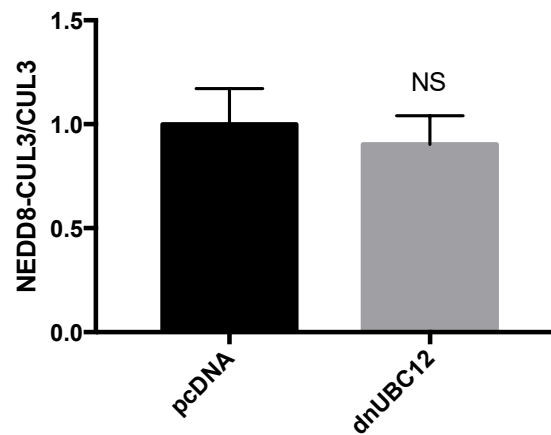
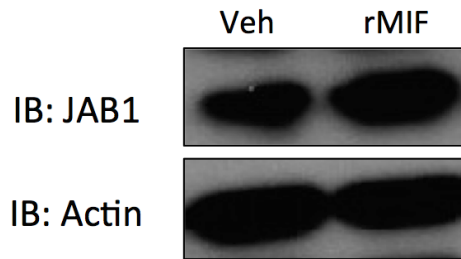


Figure 4-9: dnUBC12 expression does not alter CUL3 neddylation. RMVECs were transiently transfected with 1 μ g of either pcDNA or dnUBC12 and then harvested approximately 24 hours post-transfection. Western blot analysis of A.) CUL3, NEDD8-CUL3, actin and ectopic HA tag. Densitometric analysis of the western blot results of B.) Total CUL3 and C.) NEDD8-CUL3.

A.



B.

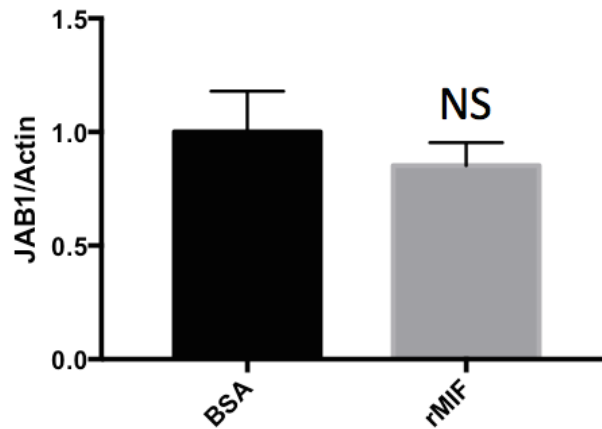
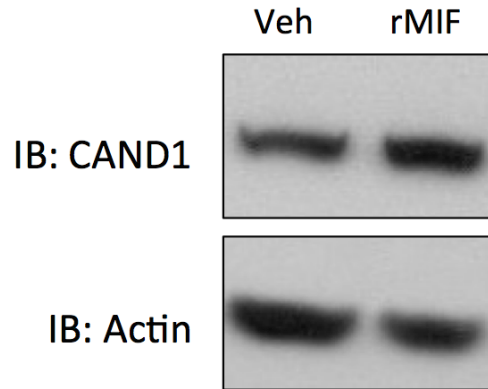
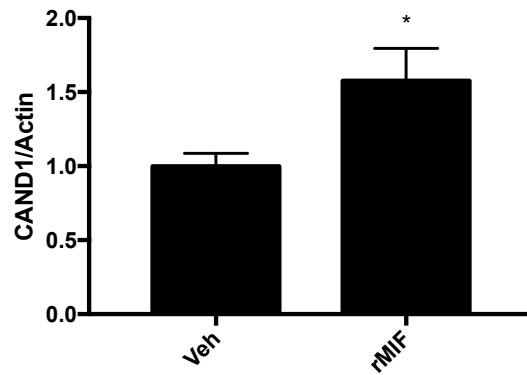


Figure 4-10: rMIF does not alter JAB1 expression *in vitro*. RMVECs were treated with either 0.1% BSA in PBS (vehicle) or rMIF (100ng/ml) for 24 hours, cells were then harvested and whole-cell lysates were analyzed by immunoblotting with A.) anti-JAB1 and anti-actin antibodies. B.) Quantification of western blot result of cells that were treated with either vehicle or rMIF for 24 hours.

A.



B.



C.

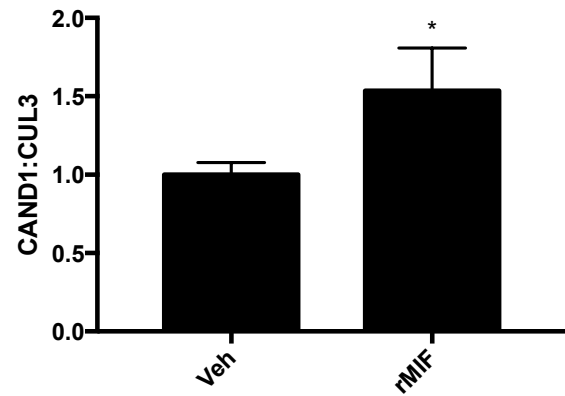
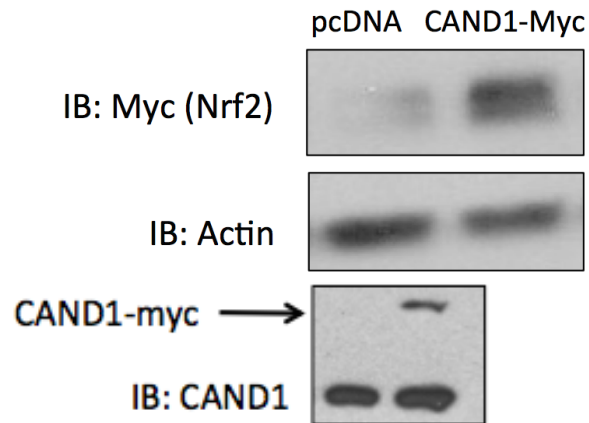


Figure 4-11: MIF-induced CAND1 expression. RMVECs were treated with either vehicle (0.1% BSA in PBS) or rMIF for 24 hours. Cells were then harvested and analyzed by immunoblotting with A.) anti-CAND1 and anti-actin antibodies B.) Densitometric quantification of the western blot result of CAND1/Actin in cells treated with either Veh or rMIF. Statistical significance is observed between Veh and rMIF treated cells as denoted by * ($p<0.05$) C.) Graphical depiction of the CAND1/CUL3 to ratio in cells treated with and without rMIF ($p=0.03$).

A.



B.

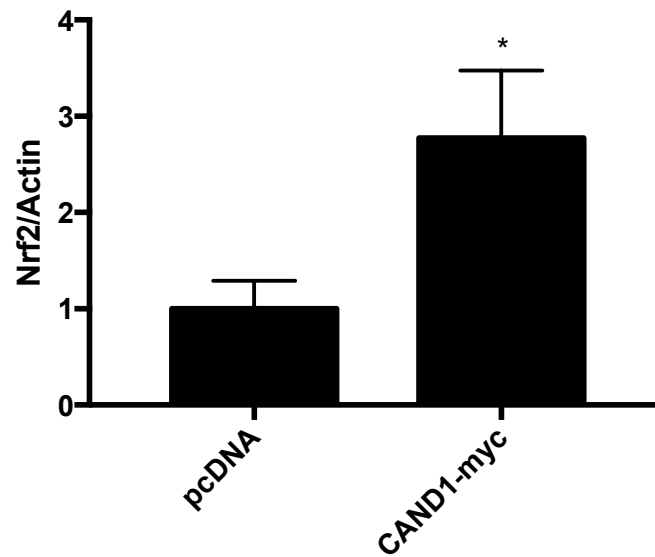


Figure 4-12: CAND1-mediated Nrf2 expression. RMVECs were co-transfected with either 0.5 ug of pcDNA or CAND1-myc and 0.5 ug of Nrf2-myc. Cells were harvested within 24 hours of transfection and whole cell lysate was analyzed with western blot A.) with anti-Myc (Nrf2), anti-CAND1 and anti-actin antibodies. B.) Densitometric quantification of myc (Nrf2) depicting a significant difference in Nrf2 expression in cells transfected with CAND1-myc (* $p < 0.05$)

Chapter 5

Role of DJ-1 in Pulmonary Endothelial Cells

5.1 Abstract

We have thus far demonstrated that MIF is an important regulator of the Nrf2-antioxidant pathway, via its ability to stabilize Nrf2 (Chapter 3) and antagonize the effects of CS-induced ROS both *in vitro* (Fig. 3-2) and *in vivo* (Damico, Simms et al. 2011, Fallica, Varela et al. 2016). We have therefore sought to determine the mechanism by which MIF is mediating Nrf2 stabilization. Nrf2 is targeted for ubiquitination by CRL3 and we have identified MIF's effects on the components of CRL3 and established that is a positive regulator of CAND1, an inhibitor of CRL3 activity. Nrf2 regulation involves the complex interactions between many molecules. DJ-1 is known to positively regulate its stability and expression in astrocytes and neuronal cells in the context of oxidative stress and Parkinson's disease (PD). DJ-1 is encoded by the *PARK7* gene is an established positive regulator of Nrf2 and antioxidant responses in the brain (Bonifati, Oostra et al. 2004).

DJ-1 is a homodimer with oxidoreductase activity that stabilizes Nrf2, upregulates Nrf2-dependent transcripts and inhibits apoptosis. Loss-of-function mutation of DJ-1 has been associated with increased ROS generation and increased susceptibility of neuronal cell death in inherited forms of PD, implicating its role as a necessary mediator of antioxidants (Bonifati, Rizzu et al. 2003). DJ-1 inhibits apoptosis by antagonizing the effects of ROS and by directly acting on pro-apoptotic pathways. It inhibits ASK1 activity in the several *in vitro* models including embryonic fibroblasts and human neuroblastoma. ASK1 activates a signaling cascade that ultimately results in apoptosis (Ouyang and Shen 2006, Mo, Jung et al. 2010). MIF was also shown to inhibit the ASK1-p38 pathway in the context of CS-induced oxidative stress (Fallica, Varela et al. 2016).

Given these functional similarities, we postulated that MIF may mediate Nrf2-dependent responses via DJ-1. We show here that DJ-1 expression is increased in the lungs of MIF deficient mice. DJ-1 overexpression however, was not sufficient to upregulate Nrf2 expression or activity in our pulmonary EC model. Moreover, DJ-1 deficiency did not sensitize these cells to the effects of CS-induced oxidative stress. Interestingly, its functions appear to be cell-type dependent, even in the context of the lung. Others have reported that DJ-1 overexpression in type II alveolar cells isolated from heavy smokers improves cell viability and enhances Nrf2-dependent transcription. We found however that DJ-1 does not appear to positively regulate Nrf2 nor antagonize CSE-induced apoptosis in our pulmonary ECs model.

5.2 Introduction

MIF is a ubiquitously expressed cytokine that was originally identified as a regulator of innate immune responses, but its functions extend far beyond the immune system (Nguyen, Beck et al. 2003, Lue, Thiele et al. 2007, Fan, Kao et al. 2014). One of its well-characterized functions is the inhibition of apoptosis by antagonizing p53 (Nguyen, Lue et al. 2003, Damico, Simms et al. 2011). MIF is cytoprotective in the context of CS-induced oxidative stress. Furthermore, there is a growing body of literature that demonstrates MIF's emerging role as a mediator of antioxidant responses (Mathew, Jacobson et al. 2013). We have also demonstrated MIF stabilizes Nrf2 by inhibiting its proteasomal-mediated degradation, via its capacity to positively regulate CAND1, a negative regulator of CUL3 (Fig. 4-11). Given MIF's effects on Nrf2 as well as its ability to antagonize ASK1 activity (Fallica, Varela et al. 2016), we have investigated the capacity of MIF to positively regulate antioxidant responses via DJ-1.

DJ-1 is a redox-sensitive, 20 kDa molecule homodimeric multifunctional protein that is conserved in both prokaryotes and eukaryotes (Hod, Penttyala et al. 1999). It was initially discovered as an oncogenic protein that promotes cell proliferation. It positively regulates Ras-mediated signaling, which is important in the cell growth and cancer development (Nagakubo, Taira et al. 1997). It is highly expressed in many tumors and its overexpression is associated with worse tumorigenic outcomes (reviewed in (Cao, Lou et al. 2015)). Furthermore, it has been shown to promote cell proliferation by antagonizing the tumor suppressor, PTEN and promoting the activation of the phosphatidylinositol-3 kinase (PI3'K)/Akt signaling pathway, which ultimately stimulates

cell growth (Kim, Peters et al. 2005). In addition to its anti-apoptotic functions, DJ-1's cytoprotective roles have been well characterized in neuronal cells and the development of PD. It acts as a molecular chaperone, preventing the aggregation of α -synuclein, as well as attenuating oxidative stress (Shendelman, Jonason et al. 2004). Furthermore, loss-of-function of the *PARK7* gene has been associated with familial early onset PD. DJ-1 inhibits PD pathogenesis by antagonizing oxidative stress and mediating antioxidant responses (Bonifati, Rizzu et al. 2003). DJ-1 loss also sensitizes cells to the effects of hydrogen peroxide *in vitro* (Taira, Saito et al. 2004). Taken together, DJ-1's role have been elucidate in the context of brain and has been shown to play an important role in antagonizing both ROS production and apoptosis.

In addition to its capacity to directly antagonize ROS, Clements et al showed that DJ-1 is indispensable for Nrf2-stability through its ability to inhibit KEAP1-mediated Nrf2 degradation in a cancer cell line. They also showed that Nrf2-dependent transcripts are attenuated in the absence of DJ-1 (Clements, McNally et al. 2006). DJ-1's effects on Nrf2 have also been shown in the context of smoking. Type II alveolar EpiCs isolated from aged smokers showed diminished DJ-1 expression and antioxidant capacity including decreased Nrf2 expression as compared to cells isolated from age-matched moderate smokers and non-smokers. They were rescued from the effects of CS-induced apoptosis by overexpression of DJ-1, implicating DJ-1 as a necessary mediator of antioxidants and cytoprotection in context of CS-induced stress in EpiCs (Bahmed, Messier et al. 2016). DJ-1's effects were also demonstrated in the context of the cancer cell line, MCF-7. The antitumor drug, BCA induces oxidant-mediated apoptosis, a phenomenon that is enhanced in the absence of DJ-1. Interestingly, sensitization of DJ-1

deficiency was independent of Nrf2, as these cells showed no changes in Nrf2-dependent transcription. Taken together, this implicates that DJ-1's ability to positively regulate Nrf2 is cell-type dependent (Ismail, Abdel Shakor et al. 2015).

In addition to its capacity to directly positively regulate Nrf2 stability, DJ-1 also regulates the Trx/ASK1 pathway (Mo, Jung et al. 2010). Reduced Trx is able to bind to ASK1, thereby inhibiting its activity. However, in the presence of oxidative stress, becomes oxidized, thereby interrupting its interaction with ASK1. This leads to phosphorylation and activation of p38 MAPK, which may result in apoptosis (Saitoh, Nishitoh et al. 1998). DJ1 can inhibit the ASK1 pathway through direct effects on ASK1 or via effects on ASK1 binding proteins, such as Trx upregulation and DAXX sequestration (Hsieh and Papaconstantinou 2006, Ouyang and Shen 2006, Im, Lee et al. 2012). In summary, while all of the functions of DJ-1 have not been elucidated, its role in antagonizing ROS-mediated injury and preventing cell death is becoming well established.

CS-induced activation of the ASK1-p38 pathway is antagonized by MIF. Fallica et al demonstrated that MIF inhibits EC apoptosis by directly antagonizing ASK1 activity and inhibiting p38 activity. They also demonstrate that ASK1 functions upstream of p38 in the context of CS-induced oxidative stress and MIF antagonizes p38 phosphorylation and p38-dependent CSE-induced apoptosis (Fallica, Varela et al. 2016). Given the functional similarities of DJ-1 and MIF, we hypothesized that MIF may impact antioxidant responses in a DJ-1-dependent manner.

5.3 Results

5.3.1 DJ-1 is Differentially Expressed in the Lung Tissues of Wild-type and MIF Deficient Mice

There is sufficient evidence demonstrating DJ-1's role in mediating cytoprotection in the context of oxidative stress by antagonizing ROS and apoptosis (Bonifati, Rizzu et al. 2003, Im, Lee et al. 2010, Bahmed, Messier et al. 2016). We have already demonstrated MIF's ability to positively regulate Nrf2 and downstream antioxidant responses. Given DJ-1's established role in mediating the Nrf2-antioxidant pathway, we hypothesize that MIF is mediating antioxidant via DJ-1. We predict that DJ-1 expression would be enhanced in context of CS and that MIF deficiency would suppress it. We therefore, investigated DJ-1 expression in the intact lung of WT and MIF^{-/-} mice exposed to either air or CS for 3 days. Mice were then harvested and the lung was homogenized for western blot analysis of DJ-1. Our results showed no differences in DJ-1 expression in MIF^{+/+} mice exposed to CS as compared to their air-exposed counterparts (Fig. 5.3.1), implicating that acute CS exposure is not sufficient to drive DJ-1 expression. Interestingly, the MIF deficient mice have enhanced DJ-1 expression as compared to their wildtype counterparts (Fig. 5.3.1), implicating that differential expression of DJ-1 is genotype-dependent and irrespective of exposure type.

5.3.2 The Loss of DJ-1 Does Not Down regulate MIF Expression

We predicted that MIF and DJ-1 are linked via their capacity to positively regulate Nrf2 we therefore, sought to elucidate DJ-1's effects on MIF expression, by abrogating DJ-1 *in vitro*. RMVECs were transfected with either on-target control siRNA

(OT) or siRNA targeted against DJ-1. We then harvested cells 72 hours post-transfection, as a previous time course revealed that this was the optimal time to ensure significant knock down of DJ-1. Whole cell lysates were run on an SDS-PAGE gel and immunoblotting was used to analyze the effects of DJ-1 loss on MIF expression. We successfully knocked down DJ-1 by about 70% (Fig. 5.3.3) ($p<0.01$), however we found no significant alterations in MIF protein expression (Fig. 5.3.3).

5.3.3 DJ-1 Expression is Not Altered *in vitro* in Response to Oxidative Stress

DJ-1 expression is enhanced in the context of ROS, which may be due to its role in antagonized oxidant-mediated stress. It can inhibit the effects of ROS both directly and indirectly by upregulating pathways that lead to Nrf2 activation (Lev, Ickowicz et al. 2008, Yanagida, Tsushima et al. 2009, Im, Lee et al. 2012). DJ-1 expression is responsive to the effects of ROS in a dose-dependent manner in other *in vitro* models (Lev, Ickowicz et al. 2008). We therefore sought to determine if increased oxidative burden would upregulate DJ-1 expression in pulmonary EC *in vitro*. We exposed RMVECs to increasing concentrations hydrogen peroxide (H_2O_2) assessed DJ-1 expression by western blotting. RMVECs were treated to 0, 0.1, 0.2 and 0.5 mMol of H_2O_2 for 24 hours. These doses were derived from a dose-response cell survival experiments. The 24-hour time point was extrapolated from previous studies that indicated sufficient time for DJ-1 to be upregulated in response to ROS generating compounds (Lev et al 2008, Mendivil-Perez et al 2014). Western blot was used to analyze DJ-1 expression in whole cell lysates. Our results reveal that 24-hour treatment with increasing H_2O_2 dose concentration failed to induce DJ-1 expression in our EC model (Fig. 5-3). We next asked if CSE would be

sufficient to induce DJ-1 expression, postulating that it would increase DJ-1 expression. We treated RMVECs with varying concentrations of cigarette smoke extract (0%, 2.5%, 5%, 7.5%, 10% and 20%) and there were no differences in DJ-1 expression in response to CSE (data not shown). Thus, exposure to H₂O₂ and CSE had no effects on absolute DJ-1 expression in EC *in vitro*.

5.3.5 DJ-1 is not Sufficient to Increase Nrf2 Expression or Activity *in vitro*

There is mounting evidence to show that DJ-1 modulates oxidative stress by mediating Nrf2 and ARE-dependent responses. DJ-1 overexpression has been shown to promote the disassociation of Nrf2 from its repressor, KEAP1 (Yan, Yang et al. 2015). Furthermore, Clements et al. showed that DJ-1's has an necessary role in stabilizing Nrf2 in a hepatocarcinoma cells. DJ-1 knockdown led to decreased Nrf2 stabilization and Nrf2-dependent transcripts (Clements, McNally et al. 2006). Furthermore, overexpression of DJ-1 restored blunted Nrf2 protein expression and Nrf2-dependent transcription in type II alveolar cells derived from aged smokers (Bahmed, Messier et al. 2016). Taken together, we postulated that overexpression of DJ-1 may be sufficient to drive both Nrf2 expression and activity in EC. We therefore, co-transfected RMVECs with Nrf2-myc plasmid and either pcDNA or myc-tagged DJ-1 plasmids under the control of the CMV promoter. Cells were harvested within 24 hours of transfection and whole cell lysates were analyzed via western blot to investigate the effects of DJ-1 on Nrf2 expression. While our cells express the DJ-1 transgene, it was not sufficient to drive Nrf2 expression in our pulmonary ECs, as there were no differences in Nrf2 expression in control cells as compared to those over expressing DJ-1 (Fig. 5-4A & 5-4B).

In a parallel experiment, we co-transfected an ARE-luciferase construct and either a pcDNA or myc-tagged DJ-1 plasmid. We then harvested cells within 24 hours and completed a luciferase assay as per manufacturer's protocol. We found no differences in ARE activity in DJ-1 overexpression as compared to empty vector transfected cells, indicating that DJ-1 is not sufficient to drive Nrf2 protein expression or ARE activity in EC *in vitro* (Fig. 5.4C).

5.3.6 DJ-1 Deficiency does not Sensitize ECs to the effects of CSE

Multiple *in vitro* models have demonstrated DJ-1's necessary role in mediating antioxidant responses and inhibiting apoptosis during oxidative stress (Clements, McNally et al. 2006, Bitar, Liu et al. 2012, Bahmed, Messier et al. 2016). The loss of DJ-1 sensitized cells to the effects of ROS-generating agents, increased ROS accumulation and inhibited antioxidant capacity (Ishiwatari, Takahashi et al. 2015, Bahmed, Messier et al. 2016). Furthermore, DJ-1 loss in types II alveolar cells derived from heavy smokers had increased markers of oxidative stress and apoptosis and decreased Nrf2-dependent transcripts. Overexpression of DJ-1 in their model decreased apoptosis and restored Nrf2-dependent responses (Bahmed et al. 2016). We therefore, postulated that the ablation of DJ-1 would sensitize RMVECs to the effects of CS. We utilized caspase 3 activity, as a marker of apoptosis. We have transfected RMVECs with either on-target control siRNA (OT) or DJ-1 siRNA and then waited 72 hours post-transfection, to ensure efficient DJ-1 knockdown treated. Cells were then treated with 20% CSE (v/v) for increased duration of time. After harvesting cells at indicated times, we assayed whole cell lysates for caspase-3 activity according to manufacturers protocol. Our results show that CSE treatment

induces a 2.5-fold increase in caspase-3 activity that peaks at around 6 hours ($p<0.01$). Interestingly, DJ-1 knockdown did not enhance CSE-induced apoptosis, as DJ-1 deficient cells showed no induction of caspase 3 activity 6 hours post-treatment (Fig. 5-5). These findings demonstrate that DJ-1 loss does not sensitize RMVECs to the effects of CSE, implicating its role is independent of antagonizing apoptosis in our ECs model in the context of oxidative stress.

5.4 Discussion & Conclusion

While we have shown that DJ-1 expression is altered in MIF deficient mice, its functions are less clear *in vivo*. We have however, determined that DJ-1 is insufficient to upregulate Nrf2 expression and ARE activity *in vitro*. Further, its loss does not enhance sensitize EC to CSE-induced apoptosis. Like MIF, DJ-1 has been demonstrated to antagonize the ASK1 pathway, which ultimately leads to p53 activation under conditions of oxidative stress (Karunakaran, Diwakar et al. 2007, Im, Lee et al. 2010, Fallica, Varela et al. 2016). Given the similarities in function, antioxidant capacities and negative regulation of the ASK1 pathway, we hypothesized that MIF and DJ-1 are interacting to inhibit EC apoptosis in the context of CS-induced oxidative stress.

Bonifati et al drew the connection between loss-of-function mutations of the PARK7 gene and familial inherited PD (Bonifati, Rizzu et al. 2003). Loss of DJ-1 function is associated with increased oxidative stress, decreased antioxidant capacity and increased mitochondrial dysfunction in the context of PD (Irrcher, Aleyasin et al. 2010, McCoy and Cookson 2011). Moreover, DJ-1 is an established positive regulator of Nrf2

in other cells types and has been demonstrated to increase Nrf2 expression, as well as downstream transcription of Nrf2-dependen targets (Yan, Yang et al. 2015, Bahmed, Messier et al. 2016). DJ-1-mediated upregulation of Nrf2 has been linked to multiple molecular mechanisms. Clements and colleagues showed that DJ-1 is necessary for Nrf2 stability and induction of Nrf2-dependent transcripts in the non-small lung cancer cell line, H157 cells (Clements, McNally et al. 2006). We therefore investigated the effects of rMIF in our cell model. Due to MIF's capacity to increase Nrf2 expression, we postulated that MIF is impacting Nrf2 stability via increased DJ-1 expression. We have shown that DJ-1 expression was upregulated in MIF^{-/-} mice, however this was not sufficient to drive the expression of Nrf2-dependent transcripts *in vivo*, as MIF^{-/-} mice had attenuated antioxidant responses to CS-induced oxidative stress that was associated with an increase in ROS accumulation in the intact lung (Fig. 3-1). Data from our lab revealed that p38 signaling is upregulated in MIF^{-/-} mice in the context of CS, which was associated with increased ROS in the intact lung (Damico, Simms et al. 2011, Fallica, Varela et al. 2016). Intriguingly, DJ-1 expression is also upregulated in these mice, which may be a phenomenon of compensation in whole body knockout of MIF. We also recognize that the lung is comprised of many different cell types, thus differential expression of DJ-1 in MIF^{-/-} may not be EC driven.

We attempted to further elucidate the potential interaction between DJ-1 and MIF by successfully knocking down DJ-1 and studying its effects on MIF expression. We found that the ablation of DJ-1 did not alter MIF expression in ECs. We have previously established that MIF antagonizes CS-induced EC apoptosis *in vitro* (Damico, Simms et

al. 2011) and *in vivo* (Fallica, Boyer et al. 2014), we asked if DJ-1 alters EC sensitivity to CSE. We also found that DJ-1 loss fails to sensitize ECs to CSE-induced apoptosis.

Previous studies have also established that DJ-1 expression is upregulated in the context of increased ROS (Lev et al 2008, Mendivil-Perez et al 2014). We therefore sought to determine if DJ-1 expression is altered by ROS in ECs *in vitro*. We found no changes in DJ-1 expression in response to increasing concentrations of H₂O₂, (concentrations 0-0.5mM) (Fig. 5-3) or CSE (data not shown). It is possible that DJ-1 represents an early redox-responsive protein and that ROS-induced DJ-1 expression is very transient or requires prolonged exposure. Given DJ-1'S role in mitigating the effects of ROS, we postulated that driving DJ-1 expression would upregulate Nrf2 expression in ECs. Our results reveal that DJ-1 overexpressing cells failed to upregulate Nrf2 expression and ARE activity. We then asked if DJ-1 was sufficient to drive both Nrf2 expression and activity *in vitro*. In a co-transfection experiment in which we overexpressed both myc-tagged Nrf2 and either an empty vector or a DJ-1 encoding plasmid, DJ-1 overexpression was not sufficient to drive Nrf2 protein expression. We also found no differences in ARE activity in DJ-1 overexpressing cells as compared to their control counterparts. Thus, in contrast to the observed effect of DJ-1 in type II EpiCs, (Bahmed, Messier et al. 2016), we have not observed a role for DJ-1 in Nrf2 and/or cytoprotection in pulmonary ECs *in vitro*.

In addition to inhibiting oxidative stress via Nrf2-dependent mechanisms, DJ-1 also inhibits the NF- κ B and MAPK signaling, thereby inhibiting phosphorylation and activation of p38 and potentiation of ROS and its downstream targets (Jo, Yeo et al. 2017). More importantly, DJ-1 expression is upregulated in many cancers, which has the

potential to drive worse disease outcomes due to its ability to promote cell viability and inhibit apoptosis (reviewed in (Cao, Lou et al. 2015)). Due to its tumor-promoting functions, serum levels of DJ-1 have the potential of being utilized as diagnostic markers for certain cancers (Benati, Montagnana et al. 2017). DJ-1's ability to inhibit apoptosis has been demonstrated in several pathways that result in the activation of the PI3K/Akt pathway (Jaramillo-Gomez, Nino et al. 2015). We therefore asked if knockdown DJ-1 would increase CS-induced apoptosis. We utilized caspase 3 activity to measure CSE-induced apoptosis. CSE significantly induced 2.5-fold increase in caspase 3 activity that was characterized by a peaked at about 6 hours post-treatment in OT transfected cells ($p<0.01$). Interestingly, this was not seen in DJ-1 deficient cells, as they were not further sensitized to the effects of CSE in the absence of DJ-1 (Fig. 5.3.6). Collectively, these data do not support a role for DJ-1 as a mediator of cytoprotection in ECs. There is a limited understanding of DJ-1's role in the lung in the setting of CS, as there is a dearth in literature about the DJ-1's function in this context. Only one study by Bahmed et al demonstrated DJ-1's cytoprotective and antioxidant-mediating capacity in epithelial type II alveolar cells (Bahmed, Messier et al. 2016). These disparate results in their *in vitro* system would suggest that DJ-1's functions are cell type-dependent.

In conclusion, we have determined that MIF can stabilize Nrf2 in ECs independent of changes in DJ-1 expression. We have also found that DJ-1 is not sufficient to increase Nrf2 expression or ARE activity, nor does its abrogation sensitize EC to the effects of CS-induced apoptosis.

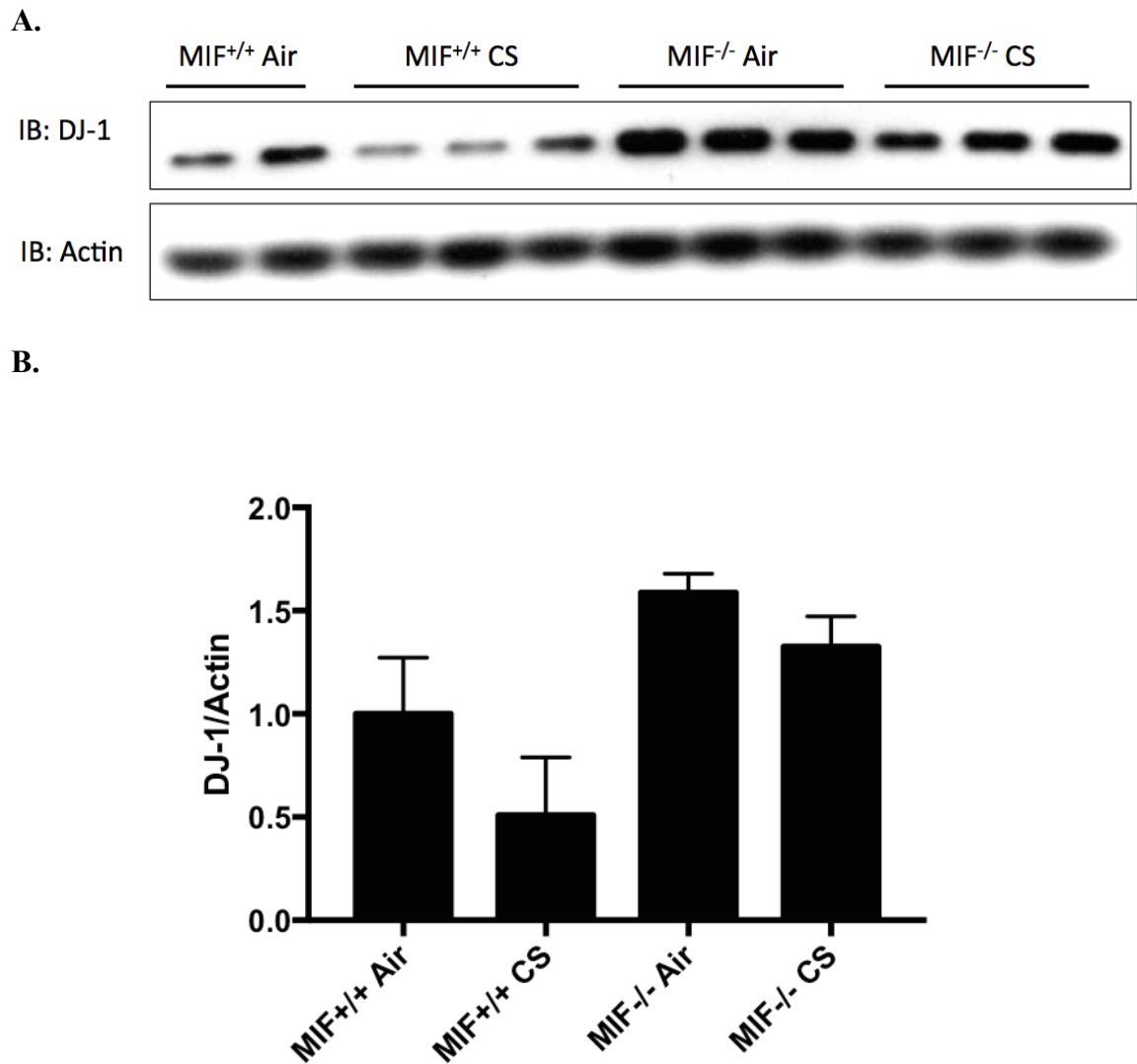


Figure 5-1: DJ-1 is upregulated in MIF^{-/-} Mice. Western blot analysis of DJ-1 protein expression in wild-type (MIF^{+/+}) and MIF deficient (MIF^{-/-}) mice exposed to either air or CS for 3 days. Right lung with excised, homogenized in cell lysis buffer and run an SDS-PAGE gel and probed with A.) anti-DJ-1 and anti-actin. B.) Densitometric analysis of western blot results. DJ-1 protein expression is increased in MIF^{-/-} mice (Two-way ANOVA: NS).

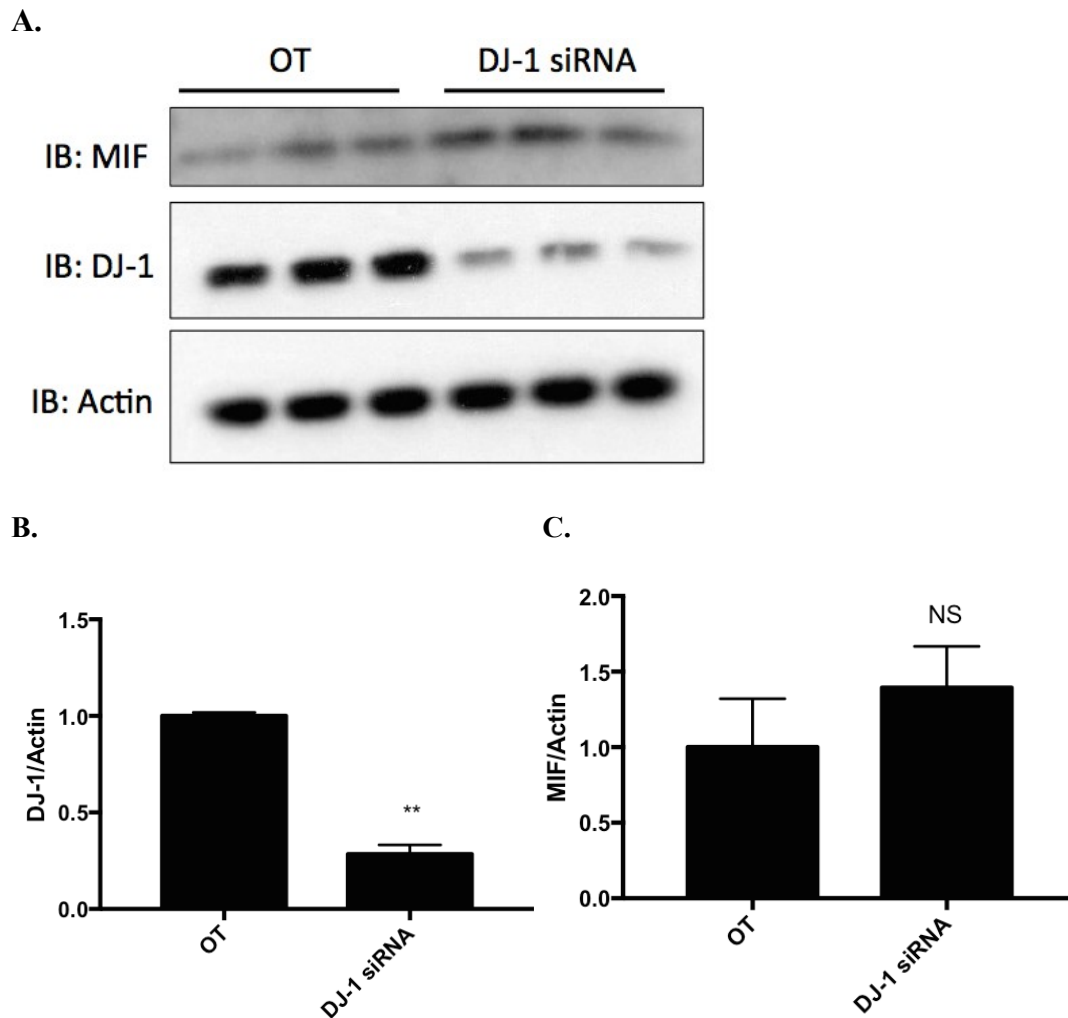


Figure 5-2: The loss of DJ-1 does not impact MIF protein expression. RMVECs were transfected with either OT siRNA or DJ-1 siRNA and were harvested 72 hours post transfection. Whole cell lysates were run an SDS-PAGE gel and probed using antibodies against A. MIF, DJ-1 and actin. B. Densitometric representation of the western blot (** $p < 0.01$).

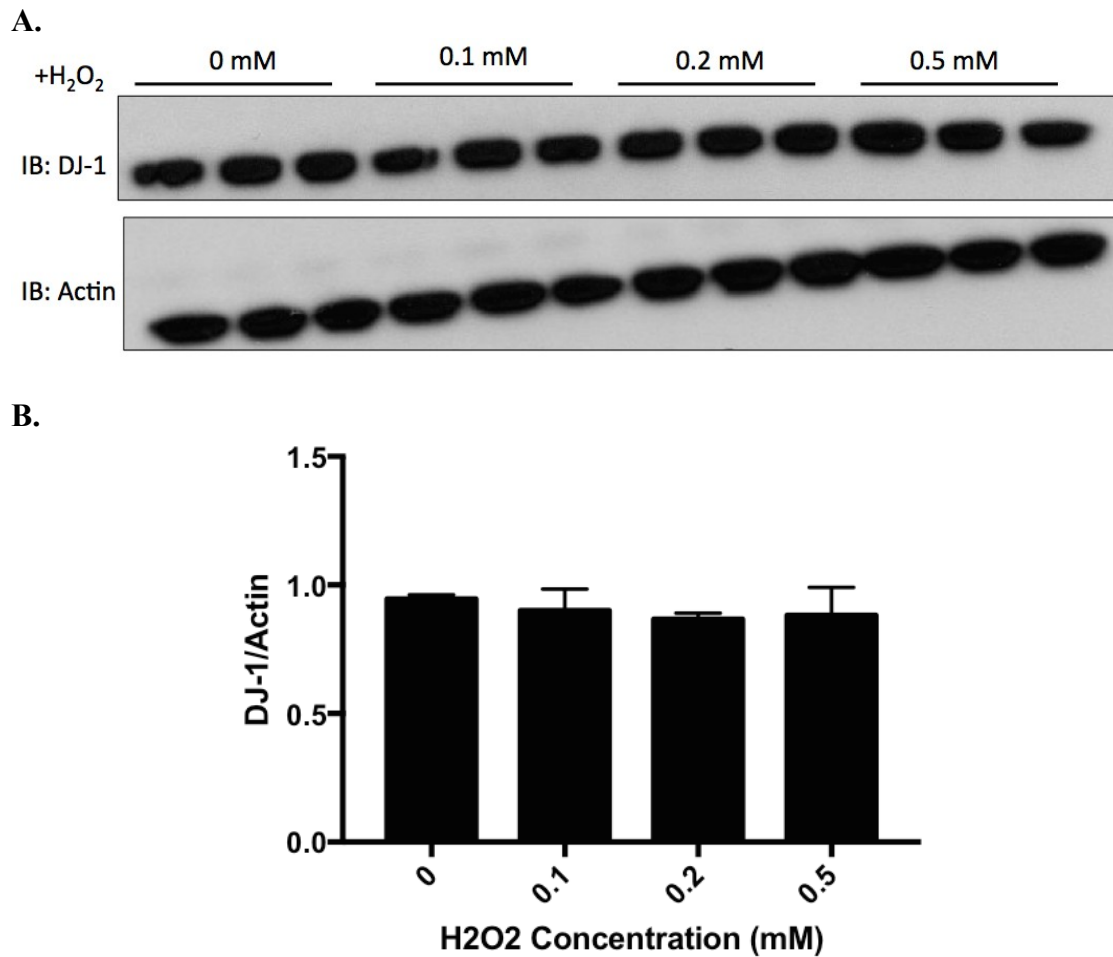
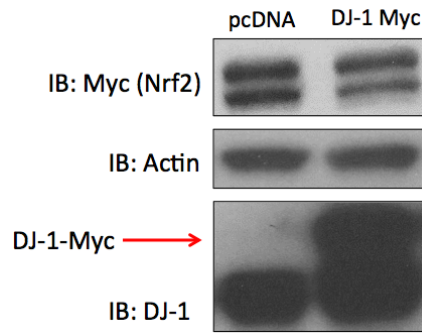
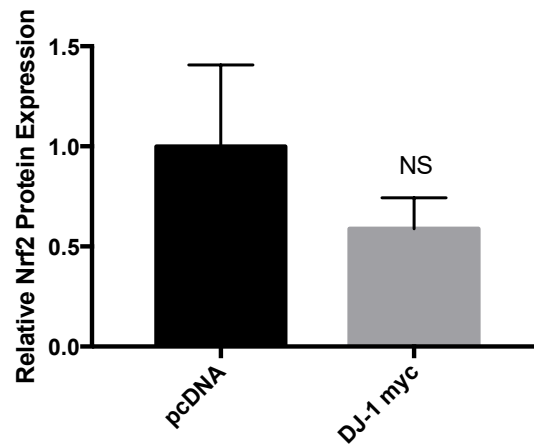


Figure 5-3: H₂O₂ does not alter DJ-1 expression in pulmonary ECs. Western blot analysis of RMVECs challenged with H₂O₂. Cells were exposed to varying concentrations of H₂O₂ (0-0.5 mM) for 24 hours, lysed and whole cell lysates were analyzed for A. DJ-1 and actin proteins expression via immunoblotting. B. Densitometric analysis of western blot.

A.



B.



C.

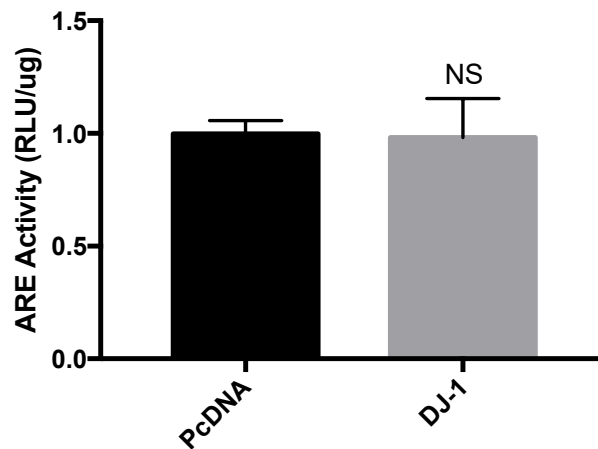
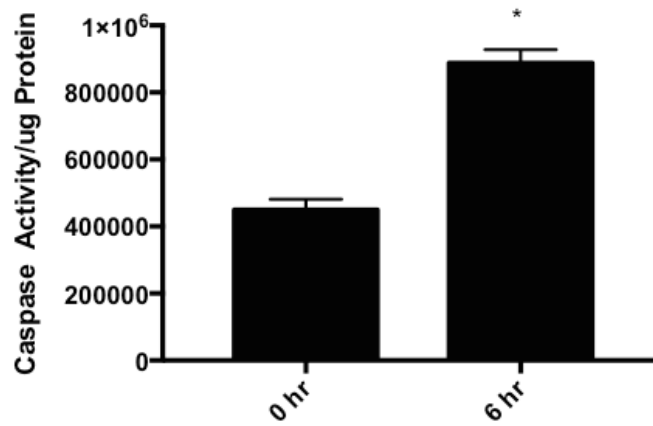


Figure 5-4: DJ-1 overexpression does not alter Nrf2 expression or ARE activity in EC *in vitro*. RMVECs were co-transfected with a plasmid encoding Nrf2-myc and either OT non-targeting siRNA or DJ-1 siRNA. Cells were harvested 72 hours -transfection. A. MIF expression was analyzed and DJ-1 knockdown was confirmed by western blot. B. Densitometric quantification of western blot results.

A.



B.

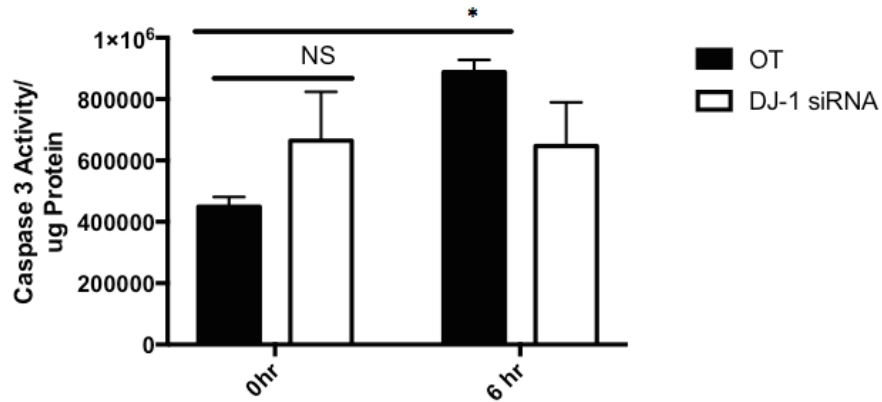


Figure 5-5: CS-Mediated Caspase 3 activity. RMVECs were transfected with either OT control or DJ-1 siRNA for 72 hours. Cells were treated with 20% CSE complete media (v/v) and harvested at 0, 2, 4, 6 and 8 hours. Caspase 3 activity assay was completed according to manufacturer's protocol A. Caspase 3 activity of cells transfected with OT (* $p < 0.05$) B. and cells transfected with DJ-1 siRNA.

Chapter 6

Thesis Summary

6.1 Summary of Thesis

COPD remains to be an important public health concern that is the third leading cause of death globally (Raheison and Girodet 2009). Emphysema, a type of COPD is characterized by permanent damage to the alveoli, leading to increased airspace size. It is a multifactorial disease that caused by many different cellular and molecular events (Sharafkhaneh, Hanania et al. 2008). EC apoptosis has been gaining recognition as an important mediator of pathogenesis. The pulmonary microvasculature is important in maintaining the structure and function of the alveolar septa. Furthermore, EC apoptosis has been identified as both a necessary and sufficient phenomenon of emphysematous tissue remodeling (Kasahara, Tuder et al. 2000, Giordano, Lahdenranta et al. 2008). Previous work from our lab has demonstrated that CS induces EC apoptosis, which is antagonized by the presence of MIF, implicating MIF's role as a necessary inhibitor of oxidant-mediated apoptosis (Damico, Simms et al. 2011). MIF serum levels as also significantly blunted in patients with advanced stage COPD, suggesting that MIF loss is associated with worse disease. Fallica et al. also showed that MIF^{-/-} mice had more severe emphysematous tissue remodeling after chronic CS exposure as compared to their wild-type counterparts (Fallica, Boyer et al. 2014). CS induces activity of the ROS-promoting enzyme, xanthine oxidoreductase (XOR) in the murine lung. Enhanced XOR activity potentiates ROS, which induces DNA double strand breaks, increased p53 activation, ultimately leading to EC apoptosis (Kim, Serebreni et al. 2013). This phenomenon is enhanced with the loss of MIF, as MIF^{-/-} mice have enhanced XOR activity during CS exposure in an ASK1-p38-dependent manner. Interestingly, in spite of displaying similar

levels of XOR activity, staining with the lipophilic ROS-sensitive dye, dihydroethidium (DHE) suggests that qualitative assessment of basal ROS may be different in the absence of MIF. These findings implicate XOR-independent and MIF-mediated mechanisms for ROS generation in the lung (Fallica, Varela et al. 2016). Taken together, we sought to understand the mechanisms by which MIF is mitigating CS-induced ROS production in pulmonary ECs.

Oxidative stress is a critical driving force in the initiation and promotion of COPD and oxidant/antioxidant homeostatic balance is disrupted in the context of disease, thus we investigated MIF's effects on antioxidant responses during CS-induced oxidative stress. We have demonstrated that MIF^{-/-} mice failed to upregulate antioxidant genes in response to acute CS exposure, demonstrating MIF's role as a necessary mediator of antioxidant responses in the lung (Fig. 3-1). We utilized two *in vitro* models, HMVECs and RMVECs to further understand how MIF is mediating antioxidant responses. We showed that pretreatment with rMIF was sufficient to antagonize CS-induced ROS accumulation in ECs (Fig. 3-2). Given MIF's capacity to drive the upregulation of antioxidant gene expression during CS exposure, we postulated that MIF's ability to mediate intracellular ROS is via the master regulator of the antioxidant response element, Nrf2. We first identified that treatment of ECs with rMIF *in vitro* was sufficient to drive activity of the DNA element, ARE (Fig. 3-3). We then showed that MIF is necessary for efficient ARE-driven response to CS exposure *in vitro* (Fig. 3-4). Given Nrf2's critical role in mediating ARE activity, we shifted our focus to understanding MIF effects on Nrf2 protein expression. We have found that rMIF significantly upregulated Nrf2 protein expression by about 2.5 fold in a proteasomal-dependent manner (Fig. 3-6). While some

mitigation of ROS *in vitro* and *in vitro* could be attributed to MIF's intrinsic oxidoreductase activity, we show here that it is a positive regulator of ARE activity, as well as the antioxidant transcription factor, Nrf2. The remainder of this thesis is aimed at determining the underlying mechanism(s) by which MIF is increasing Nrf2 expression. We thus investigated the effects of MIF on established positive and negative regulators of Nrf2.

Nrf2 is a dynamically regulated molecule with a relatively high rate of turnover. Under basal conditions, it is targeted for ubiquitination by the CRL3 ligase complex via its adaptor molecule, KEAP1 and then subsequently, degraded by the proteasome (Furukawa and Xiong 2005). KEAP1 is a redox-sensitive molecule that changes conformation in response to changes in cellular redox status (Itoh, Wakabayashi et al. 1999). Given the established function of the KEAP1-CRL3 complex in mediating Nrf2 ubiquitination, we postulated that MIF suppresses the expression or activity of components of KEAP1-CRL3. We first investigated the effects of MIF on KEAP1 and CUL3. We found that the addition of rMIF to EC in culture was not sufficient to reduce the absolute expression of either of these two molecules, as initially postulated. These data suggest that MIF's potential role in regulating the complex is independent of their expression (Fig. 4-1; 4-4). We therefore investigated MIF's effects on established regulators of CRL activity. The activity of the CRL can be modified by the covalent addition of NEDD8 to the cullin scaffold (Boh, Smith et al. 2011). This modification affects both the activity and stability of the CRL (Villeneuve, Lau et al. 2010). Our *in vivo* data showed that differential neddylation of Cul3 in lung tissues of wildtype in a MIF-dependent manner. CS significantly increased neddylation of CUL3 in the lungs of

wild type mice, which not observed in MIF deficient mice. This phenomenon however was lost in MIF^{-/-} mice, implicating MIF's role in regulating the post-translational modification of Cul3 (Fig. 4-2).

The cyclic process of neddylation and deneddylation is regulated by the enzymatic abilities of two proteins, JAB1 and UBC12 (Bosu and Kipreos 2008). JAB1, the fifth member of the COP9 signalsome has an isopeptidase activity that can cleave NEDD8 and deneddylated the complex. JAB1 is also an established binding partner to MIF. MIF has been demonstrated to negatively regulate JAB1 activity (Kleemann, Hausser et al. 2000). . Given MIF's role in negatively regulating this deneddylase, we predicted that the loss of MIF would potentiate JAB1's expression or activity. We found that MIF^{-/-} mice exposed to CS had significantly elevated JAB1 expression, which was coincident with blunted neddylation of Cul3. Furthermore, decreased Cul3 neddylation was associated with enhanced emphysematous tissue remodeling (Fallica, Boyer et al. 2014). Thee knockdown of JAB1 *in vitro* stabilized Nrf2 expression (data not shown). Given that JAB1 overexpression *in vivo* is associated with decreased neddylation of CUL3 and worse outcomes, we predicted that driving neddylation would enhance antioxidant responses. We therefore targeted UBC12 as a potential positive regulator of CUL3 neddylation and hypothesized that its overexpression would drive neddylation and stabilize Nrf2. By transiently overexpressing UBC12 in pulmonary ECs, we were able to promote CUL3 neddylation, and upregulate both, ARE activity and Nrf2 expression (Fig. 4-3). Taken together, this data shows that influencing the neddylation of CRL3 is sufficient to drive antioxidant responses both *in vivo* and *in vitro*. Changes in neddylation

potentially alter the CRL's activity or stability, ultimately inhibiting the ubiquitination of Nrf2.

Alternatively, we then sought to deneddylate the CRL complex by overexpressing a dominant negative form of UBC12 (dnUBC12) or JAB1. dnUBC12 has a point mutation that inhibits UBC12's enzymatic activity and ability to neddylation CRL. Interestingly, while we successfully overexpressed the transgene, we were unable to detect differences in the neddylation status of cells expressing dnUBC12 as compared those transfected with the pcDNA (Fig.4-7). Given that MIF deficient mice overexpressed JAB1 under conditions of CS exposure and showed diminished CUL3 neddylation, we predicted that overexpressing JAB1 in our in ECs would deneddylate CUL3. We however did not find that driving JAB1 expression altered CUL3 neddylation. Furthermore, while our *in vivo* data suggests changes in neddylation of CUL3 that may be associated with increased JAB1 expression, lung homogenates represent many different cells types. In other words, cell type other than microvascular ECs may not be driving these changes.

We have found the treatment of ECs with rMIF does not affect CUL3 neddylation, nor does it decrease JAB1 expression. We therefore, focused our efforts on studying MIF effects on other regulators of CRL3. CAND1 is an established inhibitor and regulator of the CRL3 complex. It binds to cullins, inhibiting the neddylation, activation and assembly of the complex (Dubiel, Ordemann et al. 2015). Given CAND1's role in negatively mediating CRL3, it served as an attractive target for understanding MIF-mediated regulation of the CRL. Treatment of EC's with rMIF significantly increased CAND1 expression by over 1.5 fold (Fig. 4-10). We then showed that CAND1

overexpression was sufficient to upregulate Nrf2. In summary, we demonstrate that MIF is novel positive regulator of CAND1 and may provide a mechanism by which MIF is altering CUL3 activity, ultimately stabilizing Nrf2.

This thesis aimed to investigate the effects of MIF on other regulators of Nrf2. We have found that MIF is altering the activity of an established negative regulator of Nrf2, we have also investigated MIF effects on DJ-1, a positive mediator of antioxidant responses. DJ-1 is a redox-sensitive molecule that is important in antagonizing the effects of ROS via both, Nrf2-dependent and independent mechanisms. Its role in upregulating antioxidant gene transcription has been demonstrated in neuronal cells in the context of PD (Bahmed, Messier et al. 2016). In addition to its ability to upregulate Nrf2-dependent transcripts, it also acts as a chaperone by directly binding to Nrf2, ultimately facilitating its translocation into the nucleus (Clements, McNally et al. 2006, Bitar, Liu et al. 2012). Furthermore, loss-of-function of DJ-1 is associated with increased ROS, decreased antioxidant capacity and has been linked to familial PD (Bonifati, Rizzu et al. 2003). Also similarly to MIF, DJ-1 protects against oxidative stress-induced apoptosis not only through Nrf2, but it also inhibits the activation of the ASK1-signaling pathway, which results in the activation of p53 (Im, Lee et al. 2010). Given DJ-1's role in positively regulating Nrf2 and functional similarities between MIF and DJ-1, we hypothesized that MIF may upregulate Nrf2 via a DJ-1-dependent mechanism.

We initially investigated the effects of MIF on DJ-1 expression *in vivo*. We found that DJ-1 is upregulated in the absence of MIF *in vivo*, independent of CS exposure. Given the fact that these two molecules act on the same pathways, we concluded that increases in DJ-1 expression may be the result of a compensatory mechanism, or may be

driven by other cell types in the lung. However, an increase in DJ-1 expression was not sufficient to impact antioxidant responses, because MIF^{-/-} mice still failed to upregulate Nrf2-dependent genes. We then wanted to elucidate the functional importance of DJ-1 in EC *in vitro*. We found that siRNA-mediated knockdown of DJ-1 did not further sensitize cells to the effects of CSE exposure. Furthermore, DJ-1 overexpression was not sufficient to upregulate Nrf2 expression nor ARE activity. Our data suggests that DJ-1 does not positively regulate antioxidant responses in primary pulmonary EC.

In summary, this project provides insight on MIF as a positive regulator of Nrf2, which is mediated through its ability to antagonize its proteasome-mediated degradation. We have also found that MIF acts via upregulating CAND1, an established negative regulator of CRL3.

6.2 Limitations of Work

There are several limitations to consider while interpreting these experimental results. Inherent physiological and anatomical differences between murine and human lungs pose some challenges when extrapolating results from *in vivo* models to humans, especially when attempting to model a human disease that takes decades of exposure to manifest. Additionally, the utilization of a single cell type for *in vitro* models pose important limitations as the lung itself is comprised of an interactive network of many different types of cells. There are also technical limitations to consider that may also influence interpretation of our results.

6.2.1 Limitations of In Vitro and In Vivo Models

COPD is a progressive irreversible disease with a long latency period, as most people who experience symptoms are at least 40 years of age. Given this fact, animal models of disease fail to accurately depict disease kinetics (Lokke, Lange et al. 2006), they are associated with many inherent limitations. Furthermore, rodent models do not demonstrate COPD, but rather show CS-induced emphysematous tissue remodeling. There are also fundamental anatomical and physiological differences between the pulmonary system of mice and humans. Unlike humans, mice are obligate nasal breathers. Therefore, the nasal cavity in humans is less susceptible to the effects of CS exposure and that it is unable to scrub the air entering as effectively as mice. This also implies that higher concentration of particles from CS exposure will travel further into the human bronchioles than in mice. Another important model limitation is involves using *in vitro* models to further elucidate protein function. We have employed a reductionist approach to study the effects of MIF on antioxidant responses in pulmonary ECs. While the data provides some mechanistic insight on how MIF is regulating Nrf2, it is important to recognize that this is not representative of the intact lung due to the heterogeneity of cell types that interact with one another. We have focused our studies on pulmonary ECs because of their critical role in maintaining not only the lung microvasculature, but the integrity of the alveolar septa as well. Additionally, there is a growing body of literature that demonstrates enhanced markers of EC apoptosis in patients with COPD. It has also been shown to be a determinant of emphysematous tissue remodeling *in vivo* (Giordano, Lahdenranta et al. 2008, Thomashow, Shimbo et al. 2013, Liu, Ding et al. 2014, Takahashi, Kobayashi et al. 2014). While EC apoptosis is an

important driver of remodeling, apoptosis of other cell types including EpiCs have also been implicated in disease progression (Gao, Li et al. 2015).

6.2.2. Technical Limitations

We interpret our results with careful considerations of technical limitations that may have impacted our results. One challenge that was presented to us was the identification of antibody that detects biologically-relevant Nrf2. Nrf2 exists in two isoforms, which are differentiated by their molecular weights. Nrf2-bound to ubiquitin that is detectable at around ~100 kDa and non-biologically relevant Nrf2 is detected ~70 kDa (Kemmerer, Ader et al. 2015). Detection of endogenous Nrf2 in RMVEC was not reliable. To overcome this limitation, we overexpressed ectopic Nrf2 with an epitope tag allowing us to reliably identify the transgene.

Another potential limitation is that we have focused is identifying MIF-dependent mechanisms in RMVECs *in vitro*. Although we saw the same effects of MIF on Nrf2 expression and ARE activity in both species, we have only identified MIF's effects on CAND1 in RMVECs. The major advantage of using RMVECs is that transfection efficiency of plasmid DNA is significantly more when compared to their human-donor counterparts (typically 30% vs. 5%). In spite of greater transfection efficiency in RMVECs however, the plasmid was unable to transfected in most of the cells in culture. We were still unable to deneddylate CUL3 with either JAB1 or dnUBC12 overexpression. While these findings may be true, they may also be artifacts of insufficient transfection efficiencies. In conclusion, while these models provide critical

insight into the molecular processes that regulate antioxidant responses, it is important to consider the limitations associated with them as results are interpreted.

6.3 Implications of Findings and Future Directions

In recognizing that CS, a potent source of ROS (Rennard, Decramer et al. 2002) is the leading cause of COPD in the United States, and that MIF is a determinant of emphysema *in vivo*, we have sought to determine MIF-dependent mechanisms of antioxidants regulation. In the present study, we have furthered our understanding of MIF's function in the context of CS-induced oxidative stress. Taken together, our data shows MIF's role as a mediator of antioxidant responses in the context of CS. Antioxidant capacity is depressed in patients with COPD and in smokers. Smoking cessation allows for the restoration of this protective capacity, but elevated levels of oxidative stress persist even in the absence of active CS exposure, implicating the role of diminished antioxidant capacity among other molecular processing in driving tissue destruction (Corradi, Rubinstein et al. 2003). CS has been shown to inhibit Nrf2 activity in mononuclear peripheral cells (Garbin, Fratta Pasini et al. 2009). Given the importance of oxidative stress in COPD, identifying the mechanisms that modulate Nrf2 expression and activity provides important insight into understanding disease pathogenesis. We have already identified MIF deficiency as a determinant of emphysematous tissue remodeling via its ability to impede EC apoptosis, but now shed light on its ability to positively regulate antioxidant responses in the context of CS.

Future studies should be aimed to at furthering understanding of the mechanism by which MIF is positively regulating Nrf2. We have identified MIF's novel role in stabilizing Nrf2 by antagonizing its proteasome-mediated degradation via its ability to regulate CAND1, a negative regulator of CRL. To date, expression and activity profiles of the CRL have not been well characterized in smokers and individuals with COPD. Therefore, future studies should be aimed at investigating the expression and activity profiles of these key regulators of proteasome-mediated degradation. Goven and colleagues did identify an altered Nrf2-KEAP1-Bach1 equilibrium that was coincident with a depressed antioxidant capacity phenotype in alveolar macrophages from patients with emphysema (Goven, Boutten et al. 2008). Oxidative stress is an important driver of CS-induced emphysematous remodeling and Nrf2 expression and function are important in combating the effects of CS, therefore identifying modulators of antioxidants would provide new insight in the pathogenesis of COPD.

6.4 Conclusion

In conclusion, we demonstrate that MIF is a mediator of antioxidant responses in the context of CS-induced oxidative stress. It positively regulates antioxidant response by mediating Nrf2 stability in a proteasomal-dependent mechanism. MIF stabilizes Nrf2 by upregulating the CRL complex inhibitor, CAND1 and not through the established positive Nrf2 regulator, DJ-1.

Reference

(2016). Health, United States, 2015: With Special Feature on Racial and Ethnic Health Disparities. Hyattsville, MD.

Abdel-Aleem, G. A., E. F. Khaleel, D. G. Mostafa and L. K. Elberier (2016). "Neuroprotective effect of resveratrol against brain ischemia reperfusion injury in rats entails reduction of DJ-1 protein expression and activation of PI3K/Akt/GSK3b survival pathway." Arch Physiol Biochem **122**(4): 200-213.

Adams, J., R. Kelso and L. Cooley (2000). "The kelch repeat superfamily of proteins: propellers of cell function." Trends Cell Biol **10**(1): 17-24.

Ahmad, K. F., C. K. Engel and G. G. Prive (1998). "Crystal structure of the BTB domain from PLZF." Proc Natl Acad Sci U S A **95**(21): 12123-12128.

Alber, T. (1992). "Structure of the leucine zipper." Curr Opin Genet Dev **2**(2): 205-210.

Ambroggio, X. I., D. C. Rees and R. J. Deshaies (2004). "JAMM: a metalloprotease-like zinc site in the proteasome and signalosome." PLoS Biol **2**(1): E2.

Aoshiba, K., F. Zhou, T. Tsuji and A. Nagai (2012). "DNA damage as a molecular link in the pathogenesis of COPD in smokers." Eur Respir J **39**(6): 1368-1376.

Ariga, H., K. Takahashi-Niki, I. Kato, H. Maita, T. Niki and S. M. Iguchi-Ariga (2013). "Neuroprotective function of DJ-1 in Parkinson's disease." Oxid Med Cell Longev **2013**: 683920.

Asami, S., H. Manabe, J. Miyake, Y. Tsurudome, T. Hirano, R. Yamaguchi, H. Itoh and H. Kasai (1997). "Cigarette smoking induces an increase in oxidative DNA damage, 8-hydroxydeoxyguanosine, in a central site of the human lung." Carcinogenesis **18**(9): 1763-1766.

Bacher, M., A. Meinhardt, H. Y. Lan, W. Mu, C. N. Metz, J. A. Chesney, T. Calandra, D. Gems, T. Donnelly, R. C. Atkins and R. Bucala (1997). "Migration inhibitory factor expression in experimentally induced endotoxemia." Am J Pathol **150**(1): 235-246.

Bahmed, K., E. M. Messier, W. Zhou, R. M. Tuder, C. R. Freed, H. W. Chu, S. G. Kelsen, R. P. Bowler, R. J. Mason and B. Kosmider (2016). "DJ-1 Modulates Nuclear Erythroid 2-Related Factor-2-Mediated Protection in Human Primary Alveolar Type II Cells in Smokers." Am J Respir Cell Mol Biol **55**(3): 439-449.

Balasubramanian, B., W. K. Pogozelski and T. D. Tullius (1998). "DNA strand breaking by the hydroxyl radical is governed by the accessible surface areas of the hydrogen atoms of the DNA backbone." Proc Natl Acad Sci U S A **95**(17): 9738-9743.

- Banki, K., E. Hutter, N. J. Gonchoroff and A. Perl (1999). "Elevation of mitochondrial transmembrane potential and reactive oxygen intermediate levels are early events and occur independently from activation of caspases in Fas signaling." J Immunol **162**(3): 1466-1479.
- Bannister, A. J. and T. Kouzarides (1996). "The CBP co-activator is a histone acetyltransferase." Nature **384**(6610): 641-643.
- Bardwell, V. J. and R. Treisman (1994). "The POZ domain: a conserved protein-protein interaction motif." Genes Dev **8**(14): 1664-1677.
- Barnes, N., P. M. Calverley, A. Kaplan and K. F. Rabe (2013). "Chronic obstructive pulmonary disease and exacerbations: patient insights from the global Hidden Depths of COPD survey." BMC Pulm Med **13**: 54.
- Barnes, P. J. (2009). "The cytokine network in chronic obstructive pulmonary disease." Am J Respir Cell Mol Biol **41**(6): 631-638.
- Barnes, P. J. and B. R. Celli (2009). "Systemic manifestations and comorbidities of COPD." Eur Respir J **33**(5): 1165-1185.
- Barrett, W. C., J. P. DeGnore, Y. F. Keng, Z. Y. Zhang, M. B. Yim and P. B. Chock (1999). "Roles of superoxide radical anion in signal transduction mediated by reversible regulation of protein-tyrosine phosphatase 1B." J Biol Chem **274**(49): 34543-34546.
- Benati, M., M. Montagnana, E. Danese, E. Paviati, S. Giudici, O. Ruzzenente, M. Franchi and G. Lippi (2017). "The clinical significance of DJ-1 and HE4 in patients with endometrial cancer." J Clin Lab Anal.
- Berg, J. P., Q. Zhou, K. Breuhahn, P. Schirmacher, M. A. Patil, X. Chen, N. Schafer, T. T. Holler, H. P. Fischer, R. Buttner and I. Gutgemann (2007). "Inverse expression of Jun activation domain binding protein 1 and cell cycle inhibitor p27Kip1: influence on proliferation in hepatocellular carcinoma." Hum Pathol **38**(11): 1621-1627.
- Bergin, D. A., E. P. Reeves, P. Meleady, M. Henry, O. J. McElvaney, T. P. Carroll, C. Condrón, S. H. Chotirmall, M. Clynes, S. J. O'Neill and N. G. McElvaney (2010). "alpha-1 Antitrypsin regulates human neutrophil chemotaxis induced by soluble immune complexes and IL-8." J Clin Invest **120**(12): 4236-4250.
- Bernhagen, J., T. Calandra, R. A. Mitchell, S. B. Martin, K. J. Tracey, W. Voelter, K. R. Manogue, A. Cerami and R. Bucala (1993). "MIF is a pituitary-derived cytokine that potentiates lethal endotoxaemia." Nature **365**(6448): 756-759.
- Bernstein, J. A., N. Alexis, C. Barnes, I. L. Bernstein, J. A. Bernstein, A. Nel, D. Peden, D. Diaz-Sanchez, S. M. Tarlo and P. B. Williams (2004). "Health effects of air pollution." J Allergy Clin Immunol **114**(5): 1116-1123.

- Bitar, M. S., C. Liu, A. Ziaei, Y. Chen, T. Schmedt and U. V. Jurkunas (2012). "Decline in DJ-1 and decreased nuclear translocation of Nrf2 in Fuchs endothelial corneal dystrophy." Invest Ophthalmol Vis Sci **53**(9): 5806-5813.
- Bloom, B. R. and B. Bennett (1966). "Mechanism of a reaction in vitro associated with delayed-type hypersensitivity." Science **153**(3731): 80-82.
- Bloom, J., S. Sun and Y. Al-Abed (2016). "MIF, a controversial cytokine: a review of structural features, challenges, and opportunities for drug development." Expert Opin Ther Targets **20**(12): 1463-1475.
- Boh, B. K., P. G. Smith and T. Hagen (2011). "Neddylation-induced conformational control regulates cullin RING ligase activity in vivo." J Mol Biol **409**(2): 136-145.
- Bonifati, V., B. A. Oostra and P. Heutink (2004). "Linking DJ-1 to neurodegeneration offers novel insights for understanding the pathogenesis of Parkinson's disease." J Mol Med (Berl) **82**(3): 163-174.
- Bonifati, V., P. Rizzu, F. Squitieri, E. Krieger, N. Vanacore, J. C. van Swieten, A. Brice, C. M. van Duijn, B. Oostra, G. Meco and P. Heutink (2003). "DJ-1(PARK7), a novel gene for autosomal recessive, early onset parkinsonism." Neurol Sci **24**(3): 159-160.
- Bonifati, V., P. Rizzu, M. J. van Baren, O. Schaap, G. J. Breedveld, E. Krieger, M. C. Dekker, F. Squitieri, P. Ibanez, M. Joosse, J. W. van Dongen, N. Vanacore, J. C. van Swieten, A. Brice, G. Meco, C. M. van Duijn, B. A. Oostra and P. Heutink (2003). "Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism." Science **299**(5604): 256-259.
- Borgerding, M. and H. Klus (2005). "Analysis of complex mixtures--cigarette smoke." Exp Toxicol Pathol **57 Suppl 1**: 43-73.
- Bornstein, G., D. Ganoth and A. Hershko (2006). "Regulation of neddylation and deneddylation of cullin1 in SCFSkp2 ubiquitin ligase by F-box protein and substrate." Proc Natl Acad Sci U S A **103**(31): 11515-11520.
- Bosu, D. R. and E. T. Kipreos (2008). "Cullin-RING ubiquitin ligases: global regulation and activation cycles." Cell Div **3**: 7.
- Boutten, A., D. Goven, E. Artaud-Macari, J. Boczkowski and M. Bonay (2011). "NRF2 targeting: a promising therapeutic strategy in chronic obstructive pulmonary disease." Trends Mol Med **17**(7): 363-371.
- Boveris, A., N. Oshino and B. Chance (1972). "The cellular production of hydrogen peroxide." Biochem J **128**(3): 617-630.
- Bowerman, B., B. A. Eaton and J. R. Priess (1992). "skn-1, a maternally expressed gene required to specify the fate of ventral blastomeres in the early C. elegans embryo." Cell **68**(6): 1061-1075.

- Bowler, R. P., P. J. Barnes and J. D. Crapo (2004). "The role of oxidative stress in chronic obstructive pulmonary disease." COPD **1**(2): 255-277.
- Bowler, R. P., M. Nicks, K. Tran, G. Tanner, L. Y. Chang, S. K. Young and G. S. Worthen (2004). "Extracellular superoxide dismutase attenuates lipopolysaccharide-induced neutrophilic inflammation." Am J Respir Cell Mol Biol **31**(4): 432-439.
- Bucala, R. and S. C. Donnelly (2007). "Macrophage migration inhibitory factor: a probable link between inflammation and cancer." Immunity **26**(3): 281-285.
- Burger-Kentischer, A., D. Finkelmeier, M. Thiele, J. Schmucker, G. Geiger, G. E. Tovar and J. Bernhagen (2005). "Binding of JAB1/CSN5 to MIF is mediated by the MPN domain but is independent of the JAMM motif." FEBS Lett **579**(7): 1693-1701.
- Calandra, T., J. Bernhagen, C. N. Metz, L. A. Spiegel, M. Bacher, T. Donnelly, A. Cerami and R. Bucala (1995). "MIF as a glucocorticoid-induced modulator of cytokine production." Nature **377**(6544): 68-71.
- Calandra, T., J. Bernhagen, R. A. Mitchell and R. Bucala (1994). "The macrophage is an important and previously unrecognized source of macrophage migration inhibitory factor." J Exp Med **179**(6): 1895-1902.
- Calandra, T. and T. Roger (2003). "Macrophage migration inhibitory factor: a regulator of innate immunity." Nat Rev Immunol **3**(10): 791-800.
- Calikoglu, M., A. Unlu, L. Tamer, B. Ercan, R. Bugdayci and U. Atik (2002). "The levels of serum vitamin C, malonyldialdehyde and erythrocyte reduced glutathione in chronic obstructive pulmonary disease and in healthy smokers." Clin Chem Lab Med **40**(10): 1028-1031.
- Canet-Aviles, R. M., M. A. Wilson, D. W. Miller, R. Ahmad, C. McLendon, S. Bandyopadhyay, M. J. Baptista, D. Ringe, G. A. Petsko and M. R. Cookson (2004). "The Parkinson's disease protein DJ-1 is neuroprotective due to cysteine-sulfinic acid-driven mitochondrial localization." Proc Natl Acad Sci U S A **101**(24): 9103-9108.
- Canman, C. E., D. S. Lim, K. A. Cimprich, Y. Taya, K. Tamai, K. Sakaguchi, E. Appella, M. B. Kastan and J. D. Siliciano (1998). "Activation of the ATM kinase by ionizing radiation and phosphorylation of p53." Science **281**(5383): 1677-1679.
- Canning, P., C. D. Cooper, T. Krojer, J. W. Murray, A. C. Pike, A. Chaikuad, T. Keates, C. Thangaratnarajah, V. Hojzan, V. Ayinampudi, B. D. Marsden, O. Gileadi, S. Knapp, F. von Delft and A. N. Bullock (2013). "Structural basis for Cul3 protein assembly with the BTB-Kelch family of E3 ubiquitin ligases." J Biol Chem **288**(11): 7803-7814.
- Canning, P., F. J. Sorrell and A. N. Bullock (2015). "Structural basis of Keap1 interactions with Nrf2." Free Radic Biol Med **88**(Pt B): 101-107.

Cantlay, A. M., D. Lamb, M. Gillooly, J. Norrman, D. Morrison, C. A. Smith and D. J. Harrison (1995). "Association between the CYP1A1 gene polymorphism and susceptibility to emphysema and lung cancer." Clin Mol Pathol **48**(4): M210-214.

Cao, J., S. Lou, M. Ying and B. Yang (2015). "DJ-1 as a human oncogene and potential therapeutic target." Biochem Pharmacol **93**(3): 241-250.

Celli, B. R., R. J. Halbert, R. J. Nordyke and B. Schau (2005). "Airway obstruction in never smokers: results from the Third National Health and Nutrition Examination Survey." Am J Med **118**(12): 1364-1372.

Celli, B. R., W. MacNee and A. E. T. Force (2004). "Standards for the diagnosis and treatment of patients with COPD: a summary of the ATS/ERS position paper." Eur Respir J **23**(6): 932-946.

Ceylan, E., A. Kocyigit, M. Gencer, N. Aksoy and S. Selek (2006). "Increased DNA damage in patients with chronic obstructive pulmonary disease who had once smoked or been exposed to biomass." Respir Med **100**(7): 1270-1276.

Chan, J. Y., M. C. Cheung, P. Moi, K. Chan and Y. W. Kan (1995). "Chromosomal localization of the human NF-E2 family of bZIP transcription factors by fluorescence in situ hybridization." Hum Genet **95**(3): 265-269.

Chan, K. and Y. W. Kan (1999). "Nrf2 is essential for protection against acute pulmonary injury in mice." Proc Natl Acad Sci U S A **96**(22): 12731-12736.

Chan, K., R. Lu, J. C. Chang and Y. W. Kan (1996). "NRF2, a member of the NFE2 family of transcription factors, is not essential for murine erythropoiesis, growth, and development." Proc Natl Acad Sci U S A **93**(24): 13943-13948.

Cheeseman, K. H. (1993). "Tissue injury by free radicals." Toxicol Ind Health **9**(1-2): 39-51.

Chen, C., M. Arjomandi, J. Balmes, I. Tager and N. Holland (2007). "Effects of chronic and acute ozone exposure on lipid peroxidation and antioxidant capacity in healthy young adults." Environ Health Perspect **115**(12): 1732-1737.

Cho, H. Y., S. P. Reddy and S. R. Kleeberger (2006). "Nrf2 defends the lung from oxidative stress." Antioxid Redox Signal **8**(1-2): 76-87.

Chowdhry, S., Y. Zhang, M. McMahon, C. Sutherland, A. Cuadrado and J. D. Hayes (2013). "Nrf2 is controlled by two distinct beta-TrCP recognition motifs in its Neh6 domain, one of which can be modulated by GSK-3 activity." Oncogene **32**(32): 3765-3781.

Church, D. F. and W. A. Pryor (1985). "Free-radical chemistry of cigarette smoke and its toxicological implications." Environ Health Perspect **64**: 111-126.

- Claret, F. X., M. Hibi, S. Dhut, T. Toda and M. Karin (1996). "A new group of conserved coactivators that increase the specificity of AP-1 transcription factors." Nature **383**(6599): 453-457.
- Clements, C. M., R. S. McNally, B. J. Conti, T. W. Mak and J. P. Ting (2006). "DJ-1, a cancer- and Parkinson's disease-associated protein, stabilizes the antioxidant transcriptional master regulator Nrf2." Proc Natl Acad Sci U S A **103**(41): 15091-15096.
- Cope, G. A., G. S. Suh, L. Aravind, S. E. Schwarz, S. L. Zipursky, E. V. Koonin and R. J. Deshaies (2002). "Role of predicted metalloprotease motif of Jab1/Csn5 in cleavage of Nedd8 from Cul1." Science **298**(5593): 608-611.
- Corradi, M., P. Montuschi, L. E. Donnelly, A. Pesci, S. A. Kharitonov and P. J. Barnes (2001). "Increased nitrosothiols in exhaled breath condensate in inflammatory airway diseases." Am J Respir Crit Care Med **163**(4): 854-858.
- Corradi, M., I. Rubinstein, R. Andreoli, P. Manini, A. Caglieri, D. Poli, R. Alinovi and A. Mutti (2003). "Aldehydes in exhaled breath condensate of patients with chronic obstructive pulmonary disease." Am J Respir Crit Care Med **167**(10): 1380-1386.
- Cui, D., X. Xiong and Y. Zhao (2016). "Cullin-RING ligases in regulation of autophagy." Cell Div **11**: 8.
- Cullinan, S. B., J. D. Gordan, J. Jin, J. W. Harper and J. A. Diehl (2004). "The Keap1-BTB protein is an adaptor that bridges Nrf2 to a Cul3-based E3 ligase: oxidative stress sensing by a Cul3-Keap1 ligase." Mol Cell Biol **24**(19): 8477-8486.
- Damico, R., T. Simms, B. S. Kim, Z. Tekeste, H. Amankwan, M. Damarla and P. M. Hassoun (2011). "p53 mediates cigarette smoke-induced apoptosis of pulmonary endothelial cells: inhibitory effects of macrophage migration inhibitor factor." Am J Respir Cell Mol Biol **44**(3): 323-332.
- Damico, R. L., A. Chesley, L. Johnston, E. P. Bind, E. Amaro, J. Nijmeh, B. Karakas, L. Welsh, D. B. Pearce, J. G. Garcia and M. T. Crow (2008). "Macrophage migration inhibitory factor governs endothelial cell sensitivity to LPS-induced apoptosis." Am J Respir Cell Mol Biol **39**(1): 77-85.
- Davies, K. J. (1986). "Intracellular proteolytic systems may function as secondary antioxidant defenses: an hypothesis." J Free Radic Biol Med **2**(3): 155-173.
- De Mot, R., I. Nagy and W. Baumeister (1998). "A self-compartmentalizing protease in Rhodococcus: the 20S proteasome." Antonie Van Leeuwenhoek **74**(1-3): 83-87.
- Dekhuijzen, P. N., K. K. Aben, I. Dekker, L. P. Aarts, P. L. Wielders, C. L. van Herwaarden and A. Bast (1996). "Increased exhalation of hydrogen peroxide in patients with stable and unstable chronic obstructive pulmonary disease." Am J Respir Crit Care Med **154**(3 Pt 1): 813-816.

Demedts, I. K., T. Demoor, K. R. Bracke, G. F. Joos and G. G. Brusselle (2006). "Role of apoptosis in the pathogenesis of COPD and pulmonary emphysema." Respir Res 7: 53.

Dey, T., K. Gogoi, B. G. Unni, M. Kalita, M. Bharadwaz, M. Bhattacharjee, P. K. Boruah, T. Bora, D. Ozah and M. Kalita (2014). "Role of glutathione S transferase polymorphism in COPD with special reference to peoples living in the vicinity of the open cast coal mine of Assam." PLoS One 9(5): e96739.

Dhanoa, B. S., T. Cogliati, A. G. Satish, E. A. Bruford and J. S. Friedman (2013). "Update on the Kelch-like (KLHL) gene family." Hum Genomics 7: 13.

Dinkova-Kostova, A. T., W. D. Holtzclaw, R. N. Cole, K. Itoh, N. Wakabayashi, Y. Katoh, M. Yamamoto and P. Talalay (2002). "Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants." Proc Natl Acad Sci U S A 99(18): 11908-11913.

Dobson, S. E., K. D. Augustijn, J. A. Brannigan, C. Schnick, C. J. Janse, E. J. Dodson, A. P. Waters and A. J. Wilkinson (2009). "The crystal structures of macrophage migration inhibitory factor from *Plasmodium falciparum* and *Plasmodium berghei*." Protein Sci 18(12): 2578-2591.

Donnelly, S. C. and R. Bucala (1997). "Macrophage migration inhibitory factor: a regulator of glucocorticoid activity with a critical role in inflammatory disease." Mol Med Today 3(11): 502-507.

Droge, W. (2002). "Free radicals in the physiological control of cell function." Physiol Rev 82(1): 47-95.

Du, X., I. G. Choi, R. Kim, W. Wang, J. Jancarik, H. Yokota and S. H. Kim (2000). "Crystal structure of an intracellular protease from *Pyrococcus horikoshii* at 2-Å resolution." Proc Natl Acad Sci U S A 97(26): 14079-14084.

Dubiel, D., J. Ordemann, J. Pratschke, W. Dubiel and M. Naumann (2015). "CAND1 exchange factor promotes Keap1 integration into cullin 3-RING ubiquitin ligase during adipogenesis." Int J Biochem Cell Biol 66: 95-100.

Duda, D. M., L. A. Borg, D. C. Scott, H. W. Hunt, M. Hammel and B. A. Schulman (2008). "Structural insights into NEDD8 activation of cullin-RING ligases: conformational control of conjugation." Cell 134(6): 995-1006.

Eggler, A. L., E. Small, M. Hannink and A. D. Mesecar (2009). "Cul3-mediated Nrf2 ubiquitination and antioxidant response element (ARE) activation are dependent on the partial molar volume at position 151 of Keap1." Biochem J 422(1): 171-180.

Eisner, M. D., N. Anthonisen, D. Coultas, N. Kuenzli, R. Perez-Padilla, D. Postma, I. Romieu, E. K. Silverman, J. R. Balmes, E. Committee on Nonsmoking Copd and A. Occupational Health (2010). "An official American Thoracic Society public policy

statement: Novel risk factors and the global burden of chronic obstructive pulmonary disease." Am J Respir Crit Care Med **182**(5): 693-718.

Ellenberger, T. (1994). "Getting a Grip on DNA Recognition - Structures of the Basic Region Leucine-Zipper, and the Basic Region Helix-Loop-Helix DNA-Binding Domains." Current Opinion in Structural Biology **4**(1): 12-21.

Eltoweissy, M., G. H. Dihazi, G. A. Muller, A. R. Asif and H. Dihazi (2016). "Protein DJ-1 and its anti-oxidative stress function play an important role in renal cell mediated response to profibrotic agents." Mol Biosyst **12**(6): 1842-1859.

Errington, W. J., M. Q. Khan, S. A. Bueler, J. L. Rubinstein, A. Chakrabartty and G. G. Prive (2012). "Adaptor protein self-assembly drives the control of a cullin-RING ubiquitin ligase." Structure **20**(7): 1141-1153.

Ezzati, M. and A. D. Lopez (2004). "Regional, disease specific patterns of smoking-attributable mortality in 2000." Tob Control **13**(4): 388-395.

Fallica, J., L. Boyer, B. Kim, L. Serebreni, L. Varela, O. Hamdan, L. Wang, T. Simms, M. Damarla, T. M. Kolb, R. Bucala, W. Mitzner, P. M. Hassoun and R. Damico (2014). "Macrophage migration inhibitory factor is a novel determinant of cigarette smoke-induced lung damage." Am J Respir Cell Mol Biol **51**(1): 94-103.

Fallica, J., L. Varela, L. Johnston, B. Kim, L. Serebreni, L. Wang, M. Damarla, T. M. Kolb, P. M. Hassoun and R. Damico (2016). "Macrophage Migration Inhibitory Factor: A Novel Inhibitor of Apoptosis Signal-Regulating Kinase 1-p38-Xanthine Oxidoreductase-Dependent Cigarette Smoke-Induced Apoptosis." Am J Respir Cell Mol Biol **54**(4): 504-514.

Fan, C., D. Rajasekaran, M. A. Syed, L. Leng, J. P. Loria, V. Bhandari, R. Bucala and E. J. Lolis (2013). "MIF intersubunit disulfide mutant antagonist supports activation of CD74 by endogenous MIF trimer at physiologic concentrations." Proc Natl Acad Sci U S A **110**(27): 10994-10999.

Fan, H., W. Kao, Y. H. Yang, R. Gu, J. Harris, G. Fingerle-Rowson, R. Bucala, D. Ngo, E. Beaulieu and E. F. Morand (2014). "Macrophage migration inhibitory factor inhibits the antiinflammatory effects of glucocorticoids via glucocorticoid-induced leucine zipper." Arthritis Rheumatol **66**(8): 2059-2070.

Fingerle-Rowson, G., O. Petrenko, C. N. Metz, T. G. Forsthuber, R. Mitchell, R. Huss, U. Moll, W. Muller and R. Bucala (2003). "The p53-dependent effects of macrophage migration inhibitory factor revealed by gene targeting." Proc Natl Acad Sci U S A **100**(16): 9354-9359.

Fischer, B. M., E. Pavlisko and J. A. Voynow (2011). "Pathogenic triad in COPD: oxidative stress, protease-antiprotease imbalance, and inflammation." Int J Chron Obstruct Pulmon Dis **6**: 413-421.

- Fischer, E. S., A. Scrima, K. Bohm, S. Matsumoto, G. M. Lingaraju, M. Faty, T. Yasuda, S. Cavadini, M. Wakasugi, F. Hanaoka, S. Iwai, H. Gut, K. Sugasawa and N. H. Thoma (2011). "The molecular basis of CRL4DDB2/CSA ubiquitin ligase architecture, targeting, and activation." Cell **147**(5): 1024-1039.
- Furukawa, M., Y. J. He, C. Borchers and Y. Xiong (2003). "Targeting of protein ubiquitination by BTB-Cullin 3-Roc1 ubiquitin ligases." Nat Cell Biol **5**(11): 1001-1007.
- Furukawa, M. and Y. Xiong (2005). "BTB protein Keap1 targets antioxidant transcription factor Nrf2 for ubiquitination by the Cullin 3-Roc1 ligase." Mol Cell Biol **25**(1): 162-171.
- Gan, L., D. A. Johnson and J. A. Johnson (2010). "Keap1-Nrf2 activation in the presence and absence of DJ-1." Eur J Neurosci **31**(6): 967-977.
- Gan-Erdene, T., K. Nagamalleswari, L. Yin, K. Wu, Z. Q. Pan and K. D. Wilkinson (2003). "Identification and characterization of DEN1, a deneddylase of the ULP family." J Biol Chem **278**(31): 28892-28900.
- Gao, W., L. Li, Y. Wang, S. Zhang, I. M. Adcock, P. J. Barnes, M. Huang and X. Yao (2015). "Bronchial epithelial cells: The key effector cells in the pathogenesis of chronic obstructive pulmonary disease?" Respirology **20**(5): 722-729.
- Garbin, U., A. Fratta Pasini, C. Stranieri, M. Cominacini, A. Pasini, S. Manfro, F. Lugoboni, C. Mozzini, G. Guidi, G. Faccini and L. Cominacini (2009). "Cigarette smoking blocks the protective expression of Nrf2/ARE pathway in peripheral mononuclear cells of young heavy smokers favouring inflammation." PLoS One **4**(12): e8225.
- Geboes, K., M. B. Ray, P. Rutgeerts, F. Callea, V. J. Desmet and G. Vantrappen (1982). "Morphological identification of alpha-I-antitrypsin in the human small intestine." Histopathology **6**(1): 55-60.
- Genschik, P., I. Sumara and E. Lechner (2013). "The emerging family of CULLIN3-RING ubiquitin ligases (CRL3s): cellular functions and disease implications." EMBO J **32**(17): 2307-2320.
- Gerber, H. P., A. McMurtrey, J. Kowalski, M. Yan, B. A. Keyt, V. Dixit and N. Ferrara (1998). "Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation." J Biol Chem **273**(46): 30336-30343.
- Geyer, R., S. Wee, S. Anderson, J. Yates and D. A. Wolf (2003). "BTB/POZ domain proteins are putative substrate adaptors for cullin 3 ubiquitin ligases." Mol Cell **12**(3): 783-790.
- Giordano, R. J., J. Lahdenranta, L. Zhen, U. Chukwueke, I. Petrache, R. R. Langley, I. J. Fidler, R. Pasqualini, R. M. Tudor and W. Arap (2008). "Targeted induction of lung

endothelial cell apoptosis causes emphysema-like changes in the mouse." J Biol Chem **283**(43): 29447-29460.

Goldenberg, S. J., T. C. Cascio, S. D. Shumway, K. C. Garbutt, J. Liu, Y. Xiong and N. Zheng (2004). "Structure of the Cnd1-Cul1-Roc1 complex reveals regulatory mechanisms for the assembly of the multisubunit cullin-dependent ubiquitin ligases." Cell **119**(4): 517-528.

Gong, L. and E. T. Yeh (1999). "Identification of the activating and conjugating enzymes of the NEDD8 conjugation pathway." J Biol Chem **274**(17): 12036-12042.

Goodman, R. H. and S. Smolik (2000). "CBP/p300 in cell growth, transformation, and development." Genes Dev **14**(13): 1553-1577.

Gordon, C., K. Gudi, A. Krause, R. Sackrowitz, B. G. Harvey, Y. Strulovici-Barel, J. G. Mezey and R. G. Crystal (2011). "Circulating endothelial microparticles as a measure of early lung destruction in cigarette smokers." Am J Respir Crit Care Med **184**(2): 224-232.

Goven, D., A. Boutten, V. Lecon-Malas, J. Marchal-Somme, N. Amara, B. Crestani, M. Fournier, G. Leseche, P. Soler, J. Boczkowski and M. Bonay (2008). "Altered Nrf2/Keap1-Bach1 equilibrium in pulmonary emphysema." Thorax **63**(10): 916-924.

Gunen, H., S. S. Hacievliyagil, O. Yetkin, G. Gulbas, L. C. Mutlu and E. Pehlivan (2008). "Prevalence of COPD: first epidemiological study of a large region in Turkey." Eur J Intern Med **19**(7): 499-504.

Gutteridge, J. M. (1995). "Lipid peroxidation and antioxidants as biomarkers of tissue damage." Clin Chem **41**(12 Pt 2): 1819-1828.

Hacker, S., C. Lambers, A. Pollreis, K. Hoetzenecker, M. Lichtenauer, A. Mangold, T. Niederpold, A. Hacker, G. Lang, M. Dworschak, T. Vukovich, C. Gerner, W. Klepetko and H. J. Ankersmit (2009). "Increased soluble serum markers caspase-cleaved cytokeratin-18, histones, and ST2 indicate apoptotic turnover and chronic immune response in COPD." J Clin Lab Anal **23**(6): 372-379.

Hague, S., E. Rogaeva, D. Hernandez, C. Gulick, A. Singleton, M. Hanson, J. Johnson, R. Weiser, M. Gallardo, B. Ravina, K. Gwinn-Hardy, A. Crawley, P. H. St George-Hyslop, A. E. Lang, P. Heutink, V. Bonifati, J. Hardy and A. Singleton (2003). "Early-onset Parkinson's disease caused by a compound heterozygous DJ-1 mutation." Ann Neurol **54**(2): 271-274.

He, X., G. X. Lin, M. G. Chen, J. X. Zhang and Q. Ma (2007). "Protection against chromium (VI)-induced oxidative stress and apoptosis by Nrf2. Recruiting Nrf2 into the nucleus and disrupting the nuclear Nrf2/Keap1 association." Toxicol Sci **98**(1): 298-309.

Hod, Y., S. N. Pentiyala, T. C. Whyard and M. R. El-Maghrabi (1999). "Identification and characterization of a novel protein that regulates RNA-protein interaction." J Cell Biochem **72**(3): 435-444.

- Hodge, S., G. Hodge, M. Holmes and P. N. Reynolds (2005). "Increased airway epithelial and T-cell apoptosis in COPD remains despite smoking cessation." Eur Respir J **25**(3): 447-454.
- Hogg, J. C. (2004). "Pathophysiology of airflow limitation in chronic obstructive pulmonary disease." Lancet **364**(9435): 709-721.
- Hogg, J. C., F. Chu, S. Utokaparch, R. Woods, W. M. Elliott, L. Buzatu, R. M. Cherniack, R. M. Rogers, F. C. Sciurba, H. O. Coxson and P. D. Pare (2004). "The nature of small-airway obstruction in chronic obstructive pulmonary disease." N Engl J Med **350**(26): 2645-2653.
- Hogg, J. C. and W. Timens (2009). "The pathology of chronic obstructive pulmonary disease." Annu Rev Pathol **4**: 435-459.
- Hoidal, J. R. (1994). "Pathogenesis of chronic bronchitis." Semin Respir Infect **9**(1): 8-12.
- Holland, R., A. E. Hawkins, A. L. Eggler, A. D. Mesecar, D. Fabris and J. C. Fishbein (2008). "Prospective type 1 and type 2 disulfides of Keap1 protein." Chem Res Toxicol **21**(10): 2051-2060.
- Hollingsworth, J. W., S. Maruoka, Z. Li, E. N. Potts, D. M. Brass, S. Garantziotis, A. Fong, W. M. Foster and D. A. Schwartz (2007). "Ambient ozone primes pulmonary innate immunity in mice." J Immunol **179**(7): 4367-4375.
- Honbou, K., N. N. Suzuki, M. Horiuchi, T. Taira, T. Niki, H. Ariga and F. Inagaki (2003). "Crystallization and preliminary crystallographic analysis of DJ-1, a protein associated with male fertility and parkinsonism." Acta Crystallogr D Biol Crystallogr **59**(Pt 8): 1502-1503.
- Hori, T., F. Osaka, T. Chiba, C. Miyamoto, K. Okabayashi, N. Shimbara, S. Kato and K. Tanaka (1999). "Covalent modification of all members of human cullin family proteins by NEDD8." Oncogene **18**(48): 6829-6834.
- Horvath, M. M. and N. V. Grishin (2001). "The C-terminal domain of HP1 catalase is a member of the type I glutamine amidotransferase superfamily." Proteins **42**(2): 230-236.
- Hoshino, Y. and M. Mishima (2008). "Redox-based therapeutics for lung diseases." Antioxid Redox Signal **10**(4): 701-704.
- Hsieh, C. C. and J. Papaconstantinou (2006). "Thioredoxin-ASK1 complex levels regulate ROS-mediated p38 MAPK pathway activity in livers of aged and long-lived Snell dwarf mice." FASEB J **20**(2): 259-268.
- Hua, Z. and R. D. Vierstra (2011). "The cullin-RING ubiquitin-protein ligases." Annu Rev Plant Biol **62**: 299-334.

Huang, D. T., O. Ayrault, H. W. Hunt, A. M. Taherbhoy, D. M. Duda, D. C. Scott, L. A. Borg, G. Neale, P. J. Murray, M. F. Roussel and B. A. Schulman (2009). "E2-RING expansion of the NEDD8 cascade confers specificity to cullin modification." Mol Cell **33**(4): 483-495.

Huang, X., H. D. Halicka and Z. Darzynkiewicz (2004). "Detection of histone H2AX phosphorylation on Ser-139 as an indicator of DNA damage (DNA double-strand breaks)." Curr Protoc Cytom **Chapter 7**: Unit 7 27.

Hudson, A. M. and L. Cooley (2010). "Drosophila Kelch functions with Cullin-3 to organize the ring canal actin cytoskeleton." J Cell Biol **188**(1): 29-37.

Husman, K., M. Koskenvuo, J. Kaprio, E. O. Terho and I. Vohlonen (1987). "Role of environment in the development of chronic bronchitis." Eur J Respir Dis Suppl **152**: 57-63.

Ichijo, H., E. Nishida, K. Irie, P. ten Dijke, M. Saitoh, T. Moriguchi, M. Takagi, K. Matsumoto, K. Miyazono and Y. Gotoh (1997). "Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways." Science **275**(5296): 90-94.

Iizuka, T., Y. Ishii, K. Itoh, T. Kiwamoto, T. Kimura, Y. Matsuno, Y. Morishima, A. E. Hegab, S. Homma, A. Nomura, T. Sakamoto, M. Shimura, A. Yoshida, M. Yamamoto and K. Sekizawa (2005). "Nrf2-deficient mice are highly susceptible to cigarette smoke-induced emphysema." Genes Cells **10**(12): 1113-1125.

Im, J. Y., K. W. Lee, E. Junn and M. M. Mouradian (2010). "DJ-1 protects against oxidative damage by regulating the thioredoxin/ASK1 complex." Neurosci Res **67**(3): 203-208.

Im, J. Y., K. W. Lee, J. M. Woo, E. Junn and M. M. Mouradian (2012). "DJ-1 induces thioredoxin 1 expression through the Nrf2 pathway." Hum Mol Genet **21**(13): 3013-3024.

Irrcher, I., H. Aleyasin, E. L. Seifert, S. J. Hewitt, S. Chhabra, M. Phillips, A. K. Lutz, M. W. Rousseaux, L. Bevilacqua, A. Jahani-Asl, S. Callaghan, J. G. MacLaurin, K. F. Winklhofer, P. Rizzu, P. Rippstein, R. H. Kim, C. X. Chen, E. A. Fon, R. S. Slack, M. E. Harper, H. M. McBride, T. W. Mak and D. S. Park (2010). "Loss of the Parkinson's disease-linked gene DJ-1 perturbs mitochondrial dynamics." Hum Mol Genet **19**(19): 3734-3746.

Ishii, T., T. Matsuse, S. Teramoto, H. Matsui, M. Miyao, T. Hosoi, H. Takahashi, Y. Fukuchi and Y. Ouchi (1999). "Glutathione S-transferase P1 (GSTP1) polymorphism in patients with chronic obstructive pulmonary disease." Thorax **54**(8): 693-696.

Ishiwatari, S., M. Takahashi, C. Yasuda, M. Nakagawa, Y. Saito, N. Noguchi and S. Matsukuma (2015). "The protective role of DJ-1 in ultraviolet-induced damage of human skin: DJ-1 levels in the stratum corneum as an indicator of antioxidative defense." Arch Dermatol Res **307**(10): 925-935.

Ismail, I. A., A. B. Abdel Shakor and S. H. Hong (2015). "DJ-1 Protects Breast Cancer Cells Against 2'-Benzoyloxycinnamaldehyde-induced Oxidative Stress Independent of Nrf2." J Cell Physiol **230**(9): 2262-2269.

Ito, G., H. Ariga, Y. Nakagawa and T. Iwatsubo (2006). "Roles of distinct cysteine residues in S-nitrosylation and dimerization of DJ-1." Biochem Biophys Res Commun **339**(2): 667-672.

Itoh, K., T. Chiba, S. Takahashi, T. Ishii, K. Igarashi, Y. Katoh, T. Oyake, N. Hayashi, K. Satoh, I. Hatayama, M. Yamamoto and Y. Nabeshima (1997). "An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements." Biochem Biophys Res Commun **236**(2): 313-322.

Itoh, K., T. Ishii, N. Wakabayashi and M. Yamamoto (1999). "Regulatory mechanisms of cellular response to oxidative stress." Free Radic Res **31**(4): 319-324.

Itoh, K., N. Wakabayashi, Y. Katoh, T. Ishii, K. Igarashi, J. D. Engel and M. Yamamoto (1999). "Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain." Genes Dev **13**(1): 76-86.

Izumi, T. (2003). "[Definition of chronic obstructive disease]." Nihon Rinsho **61**(12): 2058-2070.

Jan, A. T., M. Azam, K. Siddiqui, A. Ali, I. Choi and Q. M. Haq (2015). "Heavy Metals and Human Health: Mechanistic Insight into Toxicity and Counter Defense System of Antioxidants." Int J Mol Sci **16**(12): 29592-29630.

Jaramillo-Gomez, J., A. Nino, H. Arboleda and G. Arboleda (2015). "Overexpression of DJ-1 protects against C2-ceramide-induced neuronal death through activation of the PI3K/AKT pathway and inhibition of autophagy." Neurosci Lett **603**: 71-76.

Jena, N. R. (2012). "DNA damage by reactive species: Mechanisms, mutation and repair." J Biosci **37**(3): 503-517.

Jo, H. S., E. J. Yeo, M. J. Shin, Y. J. Choi, H. J. Yeo, S. B. Cho, J. H. Park, C. H. Lee, W. S. Eum and S. Y. Choi (2017). "Tat-DJ-1 enhances cell survival by inhibition of oxidative stress, NF-kappaB and MAPK activation in HepG2 cells." Biotechnol Lett **39**(4): 511-521.

Jung, H., H. A. Seong and H. Ha (2008). "Critical role of cysteine residue 81 of macrophage migration inhibitory factor (MIF) in MIF-induced inhibition of p53 activity." J Biol Chem **283**(29): 20383-20396.

Kamitani, T., K. Kito, H. P. Nguyen and E. T. Yeh (1997). "Characterization of NEDD8, a developmentally down-regulated ubiquitin-like protein." J Biol Chem **272**(45): 28557-28562.

- Kang, M. I., A. Kobayashi, N. Wakabayashi, S. G. Kim and M. Yamamoto (2004). "Scaffolding of Keap1 to the actin cytoskeleton controls the function of Nrf2 as key regulator of cytoprotective phase 2 genes." Proc Natl Acad Sci U S A **101**(7): 2046-2051.
- Kansanen, E., S. M. Kuosmanen, H. Leinonen and A. L. Levonen (2013). "The Keap1-Nrf2 pathway: Mechanisms of activation and dysregulation in cancer." Redox Biol **1**: 45-49.
- Karunakaran, S., L. Diwakar, U. Saeed, V. Agarwal, S. Ramakrishnan, S. Iyengar and V. Ravindranath (2007). "Activation of apoptosis signal regulating kinase 1 (ASK1) and translocation of death-associated protein, Daxx, in substantia nigra pars compacta in a mouse model of Parkinson's disease: protection by alpha-lipoic acid." FASEB J **21**(9): 2226-2236.
- Kasahara, Y., R. M. Tuder, C. D. Cool, D. A. Lynch, S. C. Flores and N. F. Voelkel (2001). "Endothelial cell death and decreased expression of vascular endothelial growth factor and vascular endothelial growth factor receptor 2 in emphysema." Am J Respir Crit Care Med **163**(3 Pt 1): 737-744.
- Kasahara, Y., R. M. Tuder, L. Taraseviciene-Stewart, T. D. Le Cras, S. Abman, P. K. Hirth, J. Waltenberger and N. F. Voelkel (2000). "Inhibition of VEGF receptors causes lung cell apoptosis and emphysema." J Clin Invest **106**(11): 1311-1319.
- Kato, I., H. Maita, K. Takahashi-Niki, Y. Saito, N. Noguchi, S. M. Iguchi-Ariga and H. Ariga (2013). "Oxidized DJ-1 inhibits p53 by sequestering p53 from promoters in a DNA-binding affinity-dependent manner." Mol Cell Biol **33**(2): 340-359.
- Katoh, Y., K. Iida, M. I. Kang, A. Kobayashi, M. Mizukami, K. I. Tong, M. McMahon, J. D. Hayes, K. Itoh and M. Yamamoto (2005). "Evolutionary conserved N-terminal domain of Nrf2 is essential for the Keap1-mediated degradation of the protein by proteasome." Arch Biochem Biophys **433**(2): 342-350.
- Katoh, Y., K. Itoh, E. Yoshida, M. Miyagishi, A. Fukamizu and M. Yamamoto (2001). "Two domains of Nrf2 cooperatively bind CBP, a CREB binding protein, and synergistically activate transcription." Genes Cells **6**(10): 857-868.
- Katsuoka, F., H. Motohashi, T. Ishii, H. Aburatani, J. D. Engel and M. Yamamoto (2005). "Genetic evidence that small maf proteins are essential for the activation of antioxidant response element-dependent genes." Mol Cell Biol **25**(18): 8044-8051.
- Kawakami, T., T. Chiba, T. Suzuki, K. Iwai, K. Yamanaka, N. Minato, H. Suzuki, N. Shimbara, Y. Hidaka, F. Osaka, M. Omata and K. Tanaka (2001). "NEDD8 recruits E2-ubiquitin to SCF E3 ligase." EMBO J **20**(15): 4003-4012.
- Kemmerer, Z. A., N. R. Ader, S. S. Mulroy and A. L. Eggler (2015). "Comparison of human Nrf2 antibodies: A tale of two proteins." Toxicol Lett **238**(2): 83-89.

- Kerr, J. F., A. H. Wyllie and A. R. Currie (1972). "Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics." Br J Cancer **26**(4): 239-257.
- Kim, B. S., L. Serebreni, O. Hamdan, L. Wang, A. Parniani, T. Sussan, R. Scott Stephens, L. Boyer, M. Damarla, P. M. Hassoun and R. Damico (2013). "Xanthine oxidoreductase is a critical mediator of cigarette smoke-induced endothelial cell DNA damage and apoptosis." Free Radic Biol Med **60**: 336-346.
- Kim, R. H., M. Peters, Y. Jang, W. Shi, M. Pintilie, G. C. Fletcher, C. DeLuca, J. Liepa, L. Zhou, B. Snow, R. C. Binari, A. S. Manoukian, M. R. Bray, F. F. Liu, M. S. Tsao and T. W. Mak (2005). "DJ-1, a novel regulator of the tumor suppressor PTEN." Cancer Cell **7**(3): 263-273.
- Kimura, H., Y. Sato, Y. Tajima, H. Suzuki, H. Yukitake, T. Imaeda, M. Kajino, H. Oki, M. Takizawa and S. Tanida (2010). "BTZO-1, a cardioprotective agent, reveals that macrophage migration inhibitory factor regulates ARE-mediated gene expression." Chem Biol **17**(12): 1282-1294.
- Kinumi, T., J. Kimata, T. Taira, H. Ariga and E. Niki (2004). "Cysteine-106 of DJ-1 is the most sensitive cysteine residue to hydrogen peroxide-mediated oxidation in vivo in human umbilical vein endothelial cells." Biochem Biophys Res Commun **317**(3): 722-728.
- Kirisako, T., K. Kamei, S. Murata, M. Kato, H. Fukumoto, M. Kanie, S. Sano, F. Tokunaga, K. Tanaka and K. Iwai (2006). "A ubiquitin ligase complex assembles linear polyubiquitin chains." EMBO J **25**(20): 4877-4887.
- Kirkham, P. A., G. Caramori, P. Casolari, A. A. Papi, M. Edwards, B. Shamji, K. Triantaphyllopoulos, F. Hussain, M. Pinart, Y. Khan, L. Heinemann, L. Stevens, M. Yeadon, P. J. Barnes, K. F. Chung and I. M. Adcock (2011). "Oxidative stress-induced antibodies to carbonyl-modified protein correlate with severity of chronic obstructive pulmonary disease." Am J Respir Crit Care Med **184**(7): 796-802.
- Kleemann, R., A. Hausser, G. Geiger, R. Mischke, A. Burger-Kentischer, O. Flieger, F. J. Johannes, T. Roger, T. Calandra, A. Kapurniotu, M. Grell, D. Finkelmeier, H. Brunner and J. Bernhagen (2000). "Intracellular action of the cytokine MIF to modulate AP-1 activity and the cell cycle through Jab1." Nature **408**(6809): 211-216.
- Kleemann, R., A. Kapurniotu, R. W. Frank, A. Gessner, R. Mischke, O. Flieger, S. Juttner, H. Brunner and J. Bernhagen (1998). "Disulfide analysis reveals a role for macrophage migration inhibitory factor (MIF) as thiol-protein oxidoreductase." J Mol Biol **280**(1): 85-102.
- Kleemann, R., A. Kapurniotu, R. Mischke, J. Held and J. Bernhagen (1999). "Characterization of catalytic centre mutants of macrophage migration inhibitory factor (MIF) and comparison to Cys81Ser MIF." Eur J Biochem **261**(3): 753-766.

- Kleiger, G., A. Saha, S. Lewis, B. Kuhlman and R. J. Deshaies (2009). "Rapid E2-E3 assembly and disassembly enable processive ubiquitylation of cullin-RING ubiquitin ligase substrates." Cell **139**(5): 957-968.
- Kluchova, Z., D. Petrasova, P. Joppa, Z. Dorkova and R. Tkacova (2007). "The association between oxidative stress and obstructive lung impairment in patients with COPD." Physiol Res **56**(1): 51-56.
- Kobayashi, A., M. I. Kang, H. Okawa, M. Ohtsuji, Y. Zenke, T. Chiba, K. Igarashi and M. Yamamoto (2004). "Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2." Mol Cell Biol **24**(16): 7130-7139.
- Kobayashi, M. and M. Yamamoto (2006). "Nrf2-Keap1 regulation of cellular defense mechanisms against electrophiles and reactive oxygen species." Adv Enzyme Regul **46**: 113-140.
- Koga, K., A. Kenessey, S. R. Powell, C. P. Sison, E. J. Miller and K. Ojamaa (2011). "Macrophage migration inhibitory factor provides cardioprotection during ischemia/reperfusion by reducing oxidative stress." Antioxid Redox Signal **14**(7): 1191-1202.
- Kogevinas, M., J. M. Anto, A. Tobias, J. Alonso, J. Soriano, E. Almar, N. Muniozguren, F. Payo, A. Pereira and J. Sunyer (1998). "Respiratory symptoms, lung function and use of health services among unemployed young adults in Spain. Spanish Group of the European Community Respiratory Health Survey." Eur Respir J **11**(6): 1363-1368.
- Komander, D. (2009). "The emerging complexity of protein ubiquitination." Biochem Soc Trans **37**(Pt 5): 937-953.
- Kong, A. N., E. Owuor, R. Yu, V. Hebbar, C. Chen, R. Hu and S. Mandlekar (2001). "Induction of xenobiotic enzymes by the MAP kinase pathway and the antioxidant or electrophile response element (ARE/EpRE)." Drug Metab Rev **33**(3-4): 255-271.
- Kumar, S., Y. Yoshida and M. Noda (1993). "Cloning of a cDNA which encodes a novel ubiquitin-like protein." Biochem Biophys Res Commun **195**(1): 393-399.
- Kunzli, N., R. Kaiser, S. Medina, M. Studnicka, O. Chanel, P. Filliger, M. Herry, F. Horak, Jr., V. Puybonnieux-Textier, P. Quenel, J. Schneider, R. Seethaler, J. C. Vergnaud and H. Sommer (2000). "Public-health impact of outdoor and traffic-related air pollution: a European assessment." Lancet **356**(9232): 795-801.
- Larsen, N. J., G. Ambrosi, S. J. Mullett, S. B. Berman and D. A. Hinkle (2011). "DJ-1 knock-down impairs astrocyte mitochondrial function." Neuroscience **196**: 251-264.
- Larson, R. K., M. L. Barman, F. Kueppers and H. H. Fudenberg (1970). "Genetic and environmental determinants of chronic obstructive pulmonary disease." Ann Intern Med **72**(5): 627-632.

Lee, S. J., S. J. Kim, I. K. Kim, J. Ko, C. S. Jeong, G. H. Kim, C. Park, S. O. Kang, P. G. Suh, H. S. Lee and S. S. Cha (2003). "Crystal structures of human DJ-1 and Escherichia coli Hsp31, which share an evolutionarily conserved domain." J Biol Chem **278**(45): 44552-44559.

Lee, W. and P. S. Thomas (2009). "Oxidative stress in COPD and its measurement through exhaled breath condensate." Clin Transl Sci **2**(2): 150-155.

Lee, Y. R., W. C. Yuan, H. C. Ho, C. H. Chen, H. M. Shih and R. H. Chen (2010). "The Cullin 3 substrate adaptor KLHL20 mediates DAPK ubiquitination to control interferon responses." EMBO J **29**(10): 1748-1761.

Leidecker, O., I. Matic, B. Mahata, E. Pion and D. P. Xirodimas (2012). "The ubiquitin E1 enzyme Ubel mediates NEDD8 activation under diverse stress conditions." Cell Cycle **11**(6): 1142-1150.

Leng, L., C. N. Metz, Y. Fang, J. Xu, S. Donnelly, J. Baugh, T. Delohery, Y. Chen, R. A. Mitchell and R. Bucala (2003). "MIF signal transduction initiated by binding to CD74." J Exp Med **197**(11): 1467-1476.

Lev, N., D. Ickowicz, E. Melamed and D. Offen (2008). "Oxidative insults induce DJ-1 upregulation and redistribution: implications for neuroprotection." Neurotoxicology **29**(3): 397-405.

Li, X., D. Zhang, M. Hannink and L. J. Beamer (2004). "Crystal structure of the Kelch domain of human Keap1." J Biol Chem **279**(52): 54750-54758.

Li, Y. and A. K. Jaiswal (1992). "Regulation of human NAD(P)H:quinone oxidoreductase gene. Role of AP1 binding site contained within human antioxidant response element." J Biol Chem **267**(21): 15097-15104.

Liebow, A. A. (1959). "Pulmonary emphysema with special reference to vascular changes." Am Rev Respir Dis **80**(1, Part 2): 67-93.

Liu, H., L. Ding, Y. Zhang and S. Ni (2014). "Circulating endothelial microparticles involved in lung function decline in a rat exposed in cigarette smoke maybe from apoptotic pulmonary capillary endothelial cells." J Thorac Dis **6**(6): 649-655.

Liu, J., M. Furukawa, T. Matsumoto and Y. Xiong (2002). "NEDD8 modification of CUL1 dissociates p120(CAND1), an inhibitor of CUL1-SKP1 binding and SCF ligases." Mol Cell **10**(6): 1511-1518.

Liu, J., A. Sandrini, M. C. Thurston, D. H. Yates and P. S. Thomas (2007). "Nitric oxide and exhaled breath nitrite/nitrates in chronic obstructive pulmonary disease patients." Respiration **74**(6): 617-623.

Lo, S. C. and M. Hannink (2006). "CAND1-mediated substrate adaptor recycling is required for efficient repression of Nrf2 by Keap1." Mol Cell Biol **26**(4): 1235-1244.

- Lo, S. C., X. Li, M. T. Henzl, L. J. Beamer and M. Hannink (2006). "Structure of the Keap1:Nrf2 interface provides mechanistic insight into Nrf2 signaling." EMBO J **25**(15): 3605-3617.
- Lo, Y. Y., J. M. Wong and T. F. Cruz (1996). "Reactive oxygen species mediate cytokine activation of c-Jun NH2-terminal kinases." J Biol Chem **271**(26): 15703-15707.
- Lodovici, M. and E. Bigagli (2011). "Oxidative stress and air pollution exposure." J Toxicol **2011**: 487074.
- Lokke, A., P. Lange, H. Scharling, P. Fabricius and J. Vestbo (2006). "Developing COPD: a 25 year follow up study of the general population." Thorax **61**(11): 935-939.
- Loschen, G., L. Flohe and B. Chance (1971). "Respiratory chain linked H(2)O(2) production in pigeon heart mitochondria." FEBS Lett **18**(2): 261-264.
- Louhelainen, N., P. Ryttilä, T. Haahtela, V. L. Kinnula and R. Djukanovic (2009). "Persistence of oxidant and protease burden in the airways after smoking cessation." BMC Pulm Med **9**: 25.
- Lue, H., R. Kleemann, T. Calandra, T. Roger and J. Bernhagen (2002). "Macrophage migration inhibitory factor (MIF): mechanisms of action and role in disease." Microbes Infect **4**(4): 449-460.
- Lue, H., M. Thiele, J. Franz, E. Dahl, S. Speckgens, L. Leng, G. Fingerle-Rowson, R. Bucala, B. Luscher and J. Bernhagen (2007). "Macrophage migration inhibitory factor (MIF) promotes cell survival by activation of the Akt pathway and role for CSN5/JAB1 in the control of autocrine MIF activity." Oncogene **26**(35): 5046-5059.
- Luedike, P., U. B. Hendgen-Cotta, J. Sobierajski, M. Totzeck, M. Reeh, M. Dewor, H. Lue, C. Krisp, D. Wolters, M. Kelm, J. Bernhagen and T. Rassaf (2012). "Cardioprotection through S-nitros(yl)ation of macrophage migration inhibitory factor." Circulation **125**(15): 1880-1889.
- Lyapina, S., G. Cope, A. Shevchenko, G. Serino, T. Tsuge, C. Zhou, D. A. Wolf, N. Wei, A. Shevchenko and R. J. Deshaies (2001). "Promotion of NEDD-CUL1 conjugate cleavage by COP9 signalosome." Science **292**(5520): 1382-1385.
- Lydeard, J. R., B. A. Schulman and J. W. Harper (2013). "Building and remodelling Cullin-RING E3 ubiquitin ligases." EMBO Rep **14**(12): 1050-1061.
- Ma, Q., K. Kinneer, Y. Bi, J. Y. Chan and Y. W. Kan (2004). "Induction of murine NAD(P)H:quinone oxidoreductase by 2,3,7,8-tetrachlorodibenzo-p-dioxin requires the CNC (cap 'n' collar) basic leucine zipper transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2): cross-interaction between AhR (aryl hydrocarbon receptor) and Nrf2 signal transduction." Biochem J **377**(Pt 1): 205-213.

- Mackay, L. S., S. Dodd, I. G. Dougall, W. Tomlinson, J. Lordan, A. J. Fisher and P. A. Corris (2013). "Isolation and characterisation of human pulmonary microvascular endothelial cells from patients with severe emphysema." Respir Res **14**: 23.
- MacNee, W. (2000). "Oxidants/antioxidants and COPD." Chest **117**(5 Suppl 1): 303S-317S.
- MacNee, W. (2001). "Oxidative stress and lung inflammation in airways disease." Eur J Pharmacol **429**(1-3): 195-207.
- Macnee, W. (2007). "Pathogenesis of chronic obstructive pulmonary disease." Clin Chest Med **28**(3): 479-513, v.
- Malhotra, D., E. Portales-Casamar, A. Singh, S. Srivastava, D. Arenillas, C. Happel, C. Shyr, N. Wakabayashi, T. W. Kensler, W. W. Wasserman and S. Biswal (2010). "Global mapping of binding sites for Nrf2 identifies novel targets in cell survival response through ChIP-Seq profiling and network analysis." Nucleic Acids Res **38**(17): 5718-5734.
- Man, S. F. and N. Zamel (1976). "Genetic influence on normal variability of maximum expiratory flow-volume curves." J Appl Physiol **41**(6): 874-877.
- Mannino, D. M. and A. S. Buist (2007). "Global burden of COPD: risk factors, prevalence, and future trends." Lancet **370**(9589): 765-773.
- Markson, G., C. Kiel, R. Hyde, S. Brown, P. Charalabous, A. Bremm, J. Semple, J. Woodsmith, S. Duley, K. Salehi-Ashtiani, M. Vidal, D. Komander, L. Serrano, P. Lehner and C. M. Sanderson (2009). "Analysis of the human E2 ubiquitin conjugating enzyme protein interaction network." Genome Res **19**(10): 1905-1911.
- Marnett, L. J. (1999). "Lipid peroxidation-DNA damage by malondialdehyde." Mutat Res **424**(1-2): 83-95.
- Marsh, K., E. Zaiser, P. Orfanos, S. Salverda, T. Wilcox, S. Sun and S. Dixit (2017). "Evaluation of COPD Treatments: A Multicriteria Decision Analysis of Acclidinium and Tiotropium in the United States." Value Health **20**(1): 132-140.
- Martinat, C., S. Shendelman, A. Jonason, T. Leete, M. F. Beal, L. Yang, T. Floss and A. Abeliovich (2004). "Sensitivity to oxidative stress in DJ-1-deficient dopamine neurons: an ES- derived cell model of primary Parkinsonism." PLoS Biol **2**(11): e327.
- Marwick, J. A., C. S. Stevenson, J. Giddings, W. MacNee, K. Butler, I. Rahman and P. A. Kirkham (2006). "Cigarette smoke disrupts VEGF165-VEGFR-2 receptor signaling complex in rat lungs and patients with COPD: morphological impact of VEGFR-2 inhibition." Am J Physiol Lung Cell Mol Physiol **290**(5): L897-908.
- Mathew, B., J. R. Jacobson, J. H. Siegler, J. Moitra, M. Blasco, L. Xie, C. Unzueta, T. Zhou, C. Evenoski, M. Al-Sakka, R. Sharma, B. Huey, A. Bulent, B. Smith, S.

Jayaraman, N. M. Reddy, S. P. Reddy, G. Fingerle-Rowson, R. Bucala, S. M. Dudek, V. Natarajan, R. R. Weichselbaum and J. G. Garcia (2013). "Role of migratory inhibition factor in age-related susceptibility to radiation lung injury via NF-E2-related factor-2 and antioxidant regulation." Am J Respir Cell Mol Biol **49**(2): 269-278.

McCoy, M. K. and M. R. Cookson (2011). "DJ-1 regulation of mitochondrial function and autophagy through oxidative stress." Autophagy **7**(5): 531-532.

McMahon, M., N. Thomas, K. Itoh, M. Yamamoto and J. D. Hayes (2004). "Redox-regulated turnover of Nrf2 is determined by at least two separate protein domains, the redox-sensitive Neh2 degron and the redox-insensitive Neh6 degron." J Biol Chem **279**(30): 31556-31567.

McMahon, M., N. Thomas, K. Itoh, M. Yamamoto and J. D. Hayes (2006). "Dimerization of substrate adaptors can facilitate cullin-mediated ubiquitylation of proteins by a "tethering" mechanism: a two-site interaction model for the Nrf2-Keap1 complex." J Biol Chem **281**(34): 24756-24768.

Metzger, M. B., V. A. Hristova and A. M. Weissman (2012). "HECT and RING finger families of E3 ubiquitin ligases at a glance." J Cell Sci **125**(Pt 3): 531-537.

Miao, W., L. Hu, P. J. Scrivens and G. Batist (2005). "Transcriptional regulation of NF-E2 p45-related factor (NRF2) expression by the aryl hydrocarbon receptor-xenobiotic response element signaling pathway: direct cross-talk between phase I and II drug-metabolizing enzymes." J Biol Chem **280**(21): 20340-20348.

Mischke, R., R. Kleemann, H. Brunner and J. Bernhagen (1998). "Cross-linking and mutational analysis of the oligomerization state of the cytokine macrophage migration inhibitory factor (MIF)." FEBS Lett **427**(1): 85-90.

Mitchell, R. A., C. N. Metz, T. Peng and R. Bucala (1999). "Sustained mitogen-activated protein kinase (MAPK) and cytoplasmic phospholipase A2 activation by macrophage migration inhibitory factor (MIF). Regulatory role in cell proliferation and glucocorticoid action." J Biol Chem **274**(25): 18100-18106.

Mizote, T., M. Tsuda, D. D. Smith, H. Nakayama and T. Nakazawa (1999). "Cloning and characterization of the thiD/J gene of Escherichia coli encoding a thiamin-synthesizing bifunctional enzyme, hydroxymethylpyrimidine kinase/phosphomethylpyrimidine kinase." Microbiology **145** (Pt 2): 495-501.

Mo, J. S., J. Jung, J. H. Yoon, J. A. Hong, M. Y. Kim, E. J. Ann, M. S. Seo, Y. H. Choi and H. S. Park (2010). "DJ-1 modulates the p38 mitogen-activated protein kinase pathway through physical interaction with apoptosis signal-regulating kinase 1." J Cell Biochem **110**(1): 229-237.

Mohler, J., K. Vani, S. Leung and A. Epstein (1991). "Segmentally restricted, cephalic expression of a leucine zipper gene during Drosophila embryogenesis." Mech Dev **34**(1): 3-9.

- Moi, P., K. Chan, I. Asunis, A. Cao and Y. W. Kan (1994). "Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region." Proc Natl Acad Sci U S A **91**(21): 9926-9930.
- Montuschi, P., J. V. Collins, G. Ciabattini, N. Lazzeri, M. Corradi, S. A. Kharitonov and P. J. Barnes (2000). "Exhaled 8-isoprostane as an in vivo biomarker of lung oxidative stress in patients with COPD and healthy smokers." Am J Respir Crit Care Med **162**(3 Pt 1): 1175-1177.
- Moore, D. J., L. Zhang, T. M. Dawson and V. L. Dawson (2003). "A missense mutation (L166P) in DJ-1, linked to familial Parkinson's disease, confers reduced protein stability and impairs homo-oligomerization." J Neurochem **87**(6): 1558-1567.
- Motohashi, H., F. Katsuoka, J. D. Engel and M. Yamamoto (2004). "Small Maf proteins serve as transcriptional cofactors for keratinocyte differentiation in the Keap1-Nrf2 regulatory pathway." Proc Natl Acad Sci U S A **101**(17): 6379-6384.
- Muromoto, R., M. Nakajima, K. Hirashima, T. Hirao, S. Kon, K. Shimoda, K. Oritani and T. Matsuda (2013). "Jun activation domain-binding protein 1 (JAB1) is required for the optimal response to interferons." J Biol Chem **288**(43): 30969-30979.
- Murray, C. J. and A. D. Lopez (1997). "Alternative projections of mortality and disability by cause 1990-2020: Global Burden of Disease Study." Lancet **349**(9064): 1498-1504.
- Nagakubo, D., T. Taira, H. Kitaura, M. Ikeda, K. Tamai, S. M. Iguchi-Arigo and H. Ariga (1997). "DJ-1, a novel oncogene which transforms mouse NIH3T3 cells in cooperation with ras." Biochem Biophys Res Commun **231**(2): 509-513.
- Nana-Sinkam, S. P., J. D. Lee, S. Sotto-Santiago, R. S. Stearman, R. L. Keith, Q. Choudhury, C. Cool, J. Parr, M. D. Moore, T. M. Bull, N. F. Voelkel and M. W. Geraci (2007). "Prostacyclin prevents pulmonary endothelial cell apoptosis induced by cigarette smoke." Am J Respir Crit Care Med **175**(7): 676-685.
- Nelson, S. and C. M. Mason (2000). "The inflammatory response in chronic bronchitis." Semin Respir Crit Care Med **21**(2): 79-86.
- Nguyen, M. T., J. Beck, H. Lue, H. Funzig, R. Kleemann, P. Koolwijk, A. Kapurniotu and J. Bernhagen (2003). "A 16-residue peptide fragment of macrophage migration inhibitory factor, MIF-(50-65), exhibits redox activity and has MIF-like biological functions." J Biol Chem **278**(36): 33654-33671.
- Nguyen, M. T., H. Lue, R. Kleemann, M. Thiele, G. Tolle, D. Finkelmeier, E. Wagner, A. Braun and J. Bernhagen (2003). "The cytokine macrophage migration inhibitory factor reduces pro-oxidative stress-induced apoptosis." J Immunol **170**(6): 3337-3347.

- Nguyen, T., P. Nioi and C. B. Pickett (2009). "The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress." J Biol Chem **284**(20): 13291-13295.
- Nguyen, T., P. J. Sherratt and C. B. Pickett (2003). "Regulatory mechanisms controlling gene expression mediated by the antioxidant response element." Annu Rev Pharmacol Toxicol **43**: 233-260.
- Nigro, J. M., S. J. Baker, A. C. Preisinger, J. M. Jessup, R. Hostetter, K. Cleary, S. H. Bigner, N. Davidson, S. Baylin, P. Devilee and et al. (1989). "Mutations in the p53 gene occur in diverse human tumour types." Nature **342**(6250): 705-708.
- Nioi, P., T. Nguyen, P. J. Sherratt and C. B. Pickett (2005). "The carboxy-terminal Neh3 domain of Nrf2 is required for transcriptional activation." Mol Cell Biol **25**(24): 10895-10906.
- Ohta, T., J. J. Michel and Y. Xiong (1999). "Association with cullin partners protects ROC proteins from proteasome-dependent degradation." Oncogene **18**(48): 6758-6766.
- Okawa, H., H. Motohashi, A. Kobayashi, H. Aburatani, T. W. Kensler and M. Yamamoto (2006). "Hepatocyte-specific deletion of the keap1 gene activates Nrf2 and confers potent resistance against acute drug toxicity." Biochem Biophys Res Commun **339**(1): 79-88.
- Olsen, S. K. and C. D. Lima (2013). "Structure of a ubiquitin E1-E2 complex: insights to E1-E2 thioester transfer." Mol Cell **49**(5): 884-896.
- Osaka, F., H. Kawasaki, N. Aida, M. Saeki, T. Chiba, S. Kawashima, K. Tanaka and S. Kato (1998). "A new NEDD8-ligating system for cullin-4A." Genes Dev **12**(15): 2263-2268.
- Oshikawa, K., M. Matsumoto, M. Yada, T. Kamura, S. Hatakeyama and K. I. Nakayama (2003). "Preferential interaction of TIP120A with Cull1 that is not modified by NEDD8 and not associated with Skp1." Biochem Biophys Res Commun **303**(4): 1209-1216.
- Ouyang, M. and X. Shen (2006). "Critical role of ASK1 in the 6-hydroxydopamine-induced apoptosis in human neuroblastoma SH-SY5Y cells." J Neurochem **97**(1): 234-244.
- Oyake, T., K. Itoh, H. Motohashi, N. Hayashi, H. Hoshino, M. Nishizawa, M. Yamamoto and K. Igarashi (1996). "Bach proteins belong to a novel family of BTB-basic leucine zipper transcription factors that interact with MafK and regulate transcription through the NF-E2 site." Mol Cell Biol **16**(11): 6083-6095.
- Padmanabhan, B., K. I. Tong, T. Ohta, Y. Nakamura, M. Scharlock, M. Ohtsuji, M. I. Kang, A. Kobayashi, S. Yokoyama and M. Yamamoto (2006). "Structural basis for defects of Keap1 activity provoked by its point mutations in lung cancer." Mol Cell **21**(5): 689-700.

Palange, P., U. Testa, A. Huertas, L. Calabro, R. Antonucci, E. Petrucci, E. Pelosi, L. Pasquini, A. Satta, G. Morici, M. A. Vignola and M. R. Bonsignore (2006). "Circulating haemopoietic and endothelial progenitor cells are decreased in COPD." Eur Respir J **27**(3): 529-541.

Pero, R. W., G. C. Roush, M. M. Markowitz and D. G. Miller (1990). "Oxidative stress, DNA repair, and cancer susceptibility." Cancer Detect Prev **14**(5): 555-561.

Petroski, M. D. and R. J. Deshaies (2005). "Function and regulation of cullin-RING ubiquitin ligases." Nat Rev Mol Cell Biol **6**(1): 9-20.

Phillips, A. M. (1963). "The Influence of Environmental Factors in Chronic Bronchitis." J Occup Med **5**: 468-475.

Philo, J. S., T. H. Yang and M. LaBarre (2004). "Re-examining the oligomerization state of macrophage migration inhibitory factor (MIF) in solution." Biophys Chem **108**(1-3): 77-87.

Pickart, C. M. (2001). "Mechanisms underlying ubiquitination." Annu Rev Biochem **70**: 503-533.

Pintard, L., T. Kurz, S. Glaser, J. H. Willis, M. Peter and B. Bowerman (2003). "Neddylation and deneddylation of CUL-3 is required to target MEI-1/Katanin for degradation at the meiosis-to-mitosis transition in *C. elegans*." Curr Biol **13**(11): 911-921.

Podust, V. N., J. E. Brownell, T. B. Gladysheva, R. S. Luo, C. Wang, M. B. Coggins, J. W. Pierce, E. S. Lightcap and V. Chau (2000). "A Nedd8 conjugation pathway is essential for proteolytic targeting of p27Kip1 by ubiquitination." Proc Natl Acad Sci U S A **97**(9): 4579-4584.

Pyle, M. E., M. Korbonits, M. Gueorguiev, S. Jordan, B. Kola, D. G. Morris, A. Meinhardt, M. P. Powell, F. X. Claret, Q. Zhang, C. Metz, R. Bucala and A. B. Grossman (2003). "Macrophage migration inhibitory factor expression is increased in pituitary adenoma cell nuclei." J Endocrinol **176**(1): 103-110.

Qi, D., X. Hu, X. Wu, M. Merk, L. Leng, R. Bucala and L. H. Young (2009). "Cardiac macrophage migration inhibitory factor inhibits JNK pathway activation and injury during ischemia/reperfusion." J Clin Invest **119**(12): 3807-3816.

Rachakonda, G., Y. Xiong, K. R. Sekhar, S. L. Stamer, D. C. Liebler and M. L. Freeman (2008). "Covalent modification at Cys151 dissociates the electrophile sensor Keap1 from the ubiquitin ligase CUL3." Chem Res Toxicol **21**(3): 705-710.

Rada, P., A. I. Rojo, S. Chowdhry, M. McMahon, J. D. Hayes and A. Cuadrado (2011). "SCF/ β -TrCP promotes glycogen synthase kinase 3-dependent degradation of the Nrf2 transcription factor in a Keap1-independent manner." Mol Cell Biol **31**(6): 1121-1133.

- Radjendirane, V. and A. K. Jaiswal (1999). "Antioxidant response element-mediated 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induction of human NAD(P)H:quinone oxidoreductase 1 gene expression." Biochem Pharmacol **58**(10): 1649-1655.
- Raherison, C. and P. O. Girodet (2009). "Epidemiology of COPD." Eur Respir Rev **18**(114): 213-221.
- Rahman, I. (2005). "Oxidative stress in pathogenesis of chronic obstructive pulmonary disease: cellular and molecular mechanisms." Cell Biochem Biophys **43**(1): 167-188.
- Ramirez-Carrozzi, V. R., A. A. Nazarian, C. C. Li, S. L. Gore, R. Sridharan, A. N. Imbalzano and S. T. Smale (2006). "Selective and antagonistic functions of SWI/SNF and Mi-2beta nucleosome remodeling complexes during an inflammatory response." Genes Dev **20**(3): 282-296.
- Rangasamy, T., C. Y. Cho, R. K. Thimmulappa, L. Zhen, S. S. Srisuma, T. W. Kensler, M. Yamamoto, I. Petrache, R. M. Tuder and S. Biswal (2004). "Genetic ablation of Nrf2 enhances susceptibility to cigarette smoke-induced emphysema in mice." J Clin Invest **114**(9): 1248-1259.
- Reidy, T., A. Rittenberg, M. Dwyer, S. D'Ortona, G. Pier and M. Gadjeva (2013). "Homotrimeric macrophage migration inhibitory factor (MIF) drives inflammatory responses in the corneal epithelium by promoting caveolin-rich platform assembly in response to infection." J Biol Chem **288**(12): 8269-8278.
- Reinhard, C., B. Meyer, H. Fuchs, T. Stoeger, G. Eder, F. Ruschendorf, J. Heyder, P. Nurnberg, M. H. de Angelis and H. Schulz (2005). "Genomewide linkage analysis identifies novel genetic Loci for lung function in mice." Am J Respir Crit Care Med **171**(8): 880-888.
- Rennard, S., M. Decramer, P. M. Calverley, N. B. Pride, J. B. Soriano, P. A. Vermeire and J. Vestbo (2002). "Impact of COPD in North America and Europe in 2000: subjects' perspective of Confronting COPD International Survey." Eur Respir J **20**(4): 799-805.
- Rodgers, A., M. Ezzati, S. Vander Hoorn, A. D. Lopez, R. B. Lin, C. J. Murray and G. Comparative Risk Assessment Collaborating (2004). "Distribution of major health risks: findings from the Global Burden of Disease study." PLoS Med **1**(1): e27.
- Rosengren, E., P. Aman, S. Thelin, C. Hansson, S. Ahlfors, P. Bjork, L. Jacobsson and H. Rorsman (1997). "The macrophage migration inhibitory factor MIF is a phenylpyruvate tautomerase." FEBS Lett **417**(1): 85-88.
- Rosengren, E., R. Bucala, P. Aman, L. Jacobsson, G. Odh, C. N. Metz and H. Rorsman (1996). "The immunoregulatory mediator macrophage migration inhibitory factor (MIF) catalyzes a tautomerization reaction." Mol Med **2**(1): 143-149.
- Rotin, D. and S. Kumar (2009). "Physiological functions of the HECT family of ubiquitin ligases." Nat Rev Mol Cell Biol **10**(6): 398-409.

Rubin, O., G. Canellini, J. Delobel, N. Lion and J. D. Tissot (2012). "Red blood cell microparticles: clinical relevance." Transfus Med Hemother **39**(5): 342-347.

Rushmore, T. H., R. G. King, K. E. Paulson and C. B. Pickett (1990). "Regulation of glutathione S-transferase Ya subunit gene expression: identification of a unique xenobiotic-responsive element controlling inducible expression by planar aromatic compounds." Proc Natl Acad Sci U S A **87**(10): 3826-3830.

Rushmore, T. H. and A. N. Kong (2002). "Pharmacogenomics, regulation and signaling pathways of phase I and II drug metabolizing enzymes." Curr Drug Metab **3**(5): 481-490.

Rushmore, T. H., M. R. Morton and C. B. Pickett (1991). "The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity." J Biol Chem **266**(18): 11632-11639.

Rushmore, T. H. and C. B. Pickett (1990). "Transcriptional regulation of the rat glutathione S-transferase Ya subunit gene. Characterization of a xenobiotic-responsive element controlling inducible expression by phenolic antioxidants." J Biol Chem **265**(24): 14648-14653.

Saito, R., T. Suzuki, K. Hiramoto, S. Asami, E. Naganuma, H. Suda, T. Iso, H. Yamamoto, M. Morita, L. Baird, Y. Furusawa, T. Negishi, M. Ichinose and M. Yamamoto (2015). "Characterizations of Three Major Cysteine Sensors of Keap1 in Stress Response." Mol Cell Biol **36**(2): 271-284.

Saito, Y. (2014). "Oxidized DJ-1 as a possible biomarker of Parkinson's disease." J Clin Biochem Nutr **54**(3): 138-144.

Saitoh, M., H. Nishitoh, M. Fujii, K. Takeda, K. Tobiume, Y. Sawada, M. Kawabata, K. Miyazono and H. Ichijo (1998). "Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1." EMBO J **17**(9): 2596-2606.

Salazar, M., A. I. Rojo, D. Velasco, R. M. de Sagarra and A. Cuadrado (2006). "Glycogen synthase kinase-3 β inhibits the xenobiotic and antioxidant cell response by direct phosphorylation and nuclear exclusion of the transcription factor Nrf2." J Biol Chem **281**(21): 14841-14851.

Sandford, A. J. and P. D. Pare (2000). "Genetic risk factors for chronic obstructive pulmonary disease." Clin Chest Med **21**(4): 633-643.

Sauler, M., L. Leng, M. Trentalange, M. Haslip, P. Shan, M. Piecychna, Y. Zhang, N. Andrews, P. Mannam, H. Allore, T. Fried, R. Bucala and P. J. Lee (2014). "Macrophage migration inhibitory factor deficiency in chronic obstructive pulmonary disease." Am J Physiol Lung Cell Mol Physiol **306**(6): L487-496.

Schikowski, T., D. Sugiri, U. Ranft, U. Gehring, J. Heinrich, H. E. Wichmann and U. Kramer (2005). "Long-term air pollution exposure and living close to busy roads are associated with COPD in women." Respir Res **6**: 152.

- Schmidt, E. P. and R. M. Tudor (2010). "Role of Apoptosis in Amplifying Inflammatory Responses in Lung Diseases." J Cell Death **3**: 41-53.
- Schmidt, M. W., P. R. McQuary, S. Wee, K. Hofmann and D. A. Wolf (2009). "F-box-directed CRL complex assembly and regulation by the CSN and CAND1." Mol Cell **35**(5): 586-597.
- Schulman, B. A. and J. W. Harper (2009). "Ubiquitin-like protein activation by E1 enzymes: the apex for downstream signalling pathways." Nat Rev Mol Cell Biol **10**(5): 319-331.
- Schwechheimer, C. and X. W. Deng (2001). "COP9 signalosome revisited: a novel mediator of protein degradation." Trends Cell Biol **11**(10): 420-426.
- Segura-Valdez, L., A. Pardo, M. Gaxiola, B. D. Uhal, C. Becerril and M. Selman (2000). "Upregulation of gelatinases A and B, collagenases 1 and 2, and increased parenchymal cell death in COPD." Chest **117**(3): 684-694.
- Sethi, J. M. and C. L. Rochester (2000). "Smoking and chronic obstructive pulmonary disease." Clin Chest Med **21**(1): 67-86, viii.
- Shackleford, T. J. and F. X. Claret (2010). "JAB1/CSN5: a new player in cell cycle control and cancer." Cell Div **5**: 26.
- Sharafkhaneh, A., N. A. Hanania and V. Kim (2008). "Pathogenesis of emphysema: from the bench to the bedside." Proc Am Thorac Soc **5**(4): 475-477.
- Sheehan, D., G. Meade, V. M. Foley and C. A. Dowd (2001). "Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily." Biochem J **360**(Pt 1): 1-16.
- Shendelman, S., A. Jonason, C. Martinat, T. Leete and A. Abeliovich (2004). "DJ-1 is a redox-dependent molecular chaperone that inhibits alpha-synuclein aggregate formation." PLoS Biol **2**(11): e362.
- Sies, H., C. Berndt and D. P. Jones (2017). "Oxidative Stress." Annu Rev Biochem.
- Singer, J. D., M. Gurian-West, B. Clurman and J. M. Roberts (1999). "Cullin-3 targets cyclin E for ubiquitination and controls S phase in mammalian cells." Genes Dev **13**(18): 2375-2387.
- Sirianni, F. E., F. S. Chu and D. C. Walker (2003). "Human alveolar wall fibroblasts directly link epithelial type 2 cells to capillary endothelium." Am J Respir Crit Care Med **168**(12): 1532-1537.
- Smith, L. J., M. Houston and J. Anderson (1993). "Increased levels of glutathione in bronchoalveolar lavage fluid from patients with asthma." Am Rev Respir Dis **147**(6 Pt 1): 1461-1464.

- Soucy, T. A., P. G. Smith and M. Rolfe (2009). "Targeting NEDD8-activated cullin-RING ligases for the treatment of cancer." Clin Cancer Res **15**(12): 3912-3916.
- Souphron, J., M. B. Waddell, A. Paydar, Z. Tokgoz-Gromley, M. F. Roussel and B. A. Schulman (2008). "Structural dissection of a gating mechanism preventing misactivation of ubiquitin by NEDD8's E1." Biochemistry **47**(34): 8961-8969.
- Stogios, P. J., G. S. Downs, J. J. Jauhal, S. K. Nandra and G. G. Prive (2005). "Sequence and structural analysis of BTB domain proteins." Genome Biol **6**(10): R82.
- Stogios, P. J. and G. G. Prive (2004). "The BACK domain in BTB-kelch proteins." Trends Biochem Sci **29**(12): 634-637.
- Strulovici-Barel, Y., M. R. Staudt, A. Krause, C. Gordon, A. E. Tilley, B. G. Harvey, R. J. Kaner, C. Hollmann, J. G. Mezey, H. Bitter, S. G. Pillai, H. Hilton, G. Wolff, C. S. Stevenson, S. Visvanathan, J. S. Fine and R. G. Crystal (2016). "Persistence of circulating endothelial microparticles in COPD despite smoking cessation." Thorax **71**(12): 1137-1144.
- Sumara, I., M. Quadroni, C. Frei, M. H. Olma, G. Sumara, R. Ricci and M. Peter (2007). "A Cul3-based E3 ligase removes Aurora B from mitotic chromosomes, regulating mitotic progression and completion of cytokinesis in human cells." Dev Cell **12**(6): 887-900.
- Sun, H. W., J. Bernhagen, R. Bucala and E. Lolis (1996). "Crystal structure at 2.6-A resolution of human macrophage migration inhibitory factor." Proc Natl Acad Sci U S A **93**(11): 5191-5196.
- Suzuki, M., T. Betsuyaku, Y. Ito, K. Nagai, Y. Nasuhara, K. Kaga, S. Kondo and M. Nishimura (2008). "Down-regulated NF-E2-related factor 2 in pulmonary macrophages of aged smokers and patients with chronic obstructive pulmonary disease." Am J Respir Cell Mol Biol **39**(6): 673-682.
- Swope, M., H. W. Sun, P. R. Blake and E. Lolis (1998). "Direct link between cytokine activity and a catalytic site for macrophage migration inhibitory factor." EMBO J **17**(13): 3534-3541.
- Taguchi, K., H. Motohashi and M. Yamamoto (2011). "Molecular mechanisms of the Keap1-Nrf2 pathway in stress response and cancer evolution." Genes Cells **16**(2): 123-140.
- Taira, T., Y. Saito, T. Niki, S. M. Iguchi-Ariga, K. Takahashi and H. Ariga (2004). "DJ-1 has a role in antioxidative stress to prevent cell death." EMBO Rep **5**(2): 213-218.
- Takahashi, M., J. Nishihira, M. Shimpo, Y. Mizue, S. Ueno, H. Mano, E. Kobayashi, U. Ikeda and K. Shimada (2001). "Macrophage migration inhibitory factor as a redox-sensitive cytokine in cardiac myocytes." Cardiovasc Res **52**(3): 438-445.

Takahashi, T., S. Kobayashi, N. Fujino, T. Suzuki, C. Ota, M. He, M. Yamada, S. Suzuki, M. Yanai, S. Kurosawa, M. Yamaya and H. Kubo (2012). "Increased circulating endothelial microparticles in COPD patients: a potential biomarker for COPD exacerbation susceptibility." Thorax **67**(12): 1067-1074.

Takahashi, T., S. Kobayashi, N. Fujino, T. Suzuki, C. Ota, Y. Tando, M. Yamada, M. Yanai, M. Yamaya, S. Kurosawa, M. Yamauchi and H. Kubo (2014). "Annual FEV1 changes and numbers of circulating endothelial microparticles in patients with COPD: a prospective study." BMJ Open **4**(3): e004571.

Takaya, K., T. Suzuki, H. Motohashi, K. Onodera, S. Satomi, T. W. Kensler and M. Yamamoto (2012). "Validation of the multiple sensor mechanism of the Keap1-Nrf2 system." Free Radic Biol Med **53**(4): 817-827.

Talhout, R., T. Schulz, E. Florek, J. van Benthem, P. Wester and A. Opperhuizen (2011). "Hazardous compounds in tobacco smoke." Int J Environ Res Public Health **8**(2): 613-628.

Tateishi, K., M. Omata, K. Tanaka and T. Chiba (2001). "The NEDD8 system is essential for cell cycle progression and morphogenetic pathway in mice." J Cell Biol **155**(4): 571-579.

Tavilani, H., E. Nadi, J. Karimi and M. T. Goodarzi (2012). "Oxidative stress in COPD patients, smokers, and non-smokers." Respir Care **57**(12): 2090-2094.

Thannickal, V. J. and B. L. Fanburg (2000). "Reactive oxygen species in cell signaling." Am J Physiol Lung Cell Mol Physiol **279**(6): L1005-1028.

Thomashow, M. A., D. Shimbo, M. A. Parikh, E. A. Hoffman, J. Vogel-Claussen, K. Hueper, J. Fu, C. Y. Liu, D. A. Bluemke, C. E. Ventetuolo, M. F. Doyle and R. G. Barr (2013). "Endothelial microparticles in mild chronic obstructive pulmonary disease and emphysema. The Multi-Ethnic Study of Atherosclerosis Chronic Obstructive Pulmonary Disease study." Am J Respir Crit Care Med **188**(1): 60-68.

Tkachev, V. O., E. B. Menshchikova and N. K. Zenkov (2011). "Mechanism of the Nrf2/Keap1/ARE signaling system." Biochemistry (Mosc) **76**(4): 407-422.

Tong, K. I., Y. Katoh, H. Kusunoki, K. Itoh, T. Tanaka and M. Yamamoto (2006). "Keap1 recruits Neh2 through binding to ETGE and DLG motifs: characterization of the two-site molecular recognition model." Mol Cell Biol **26**(8): 2887-2900.

Tong, K. I., A. Kobayashi, F. Katsuoka and M. Yamamoto (2006). "Two-site substrate recognition model for the Keap1-Nrf2 system: a hinge and latch mechanism." Biol Chem **387**(10-11): 1311-1320.

Tran, H. J., M. D. Allen, J. Lowe and M. Bycroft (2003). "Structure of the Jab1/MPN domain and its implications for proteasome function." Biochemistry **42**(39): 11460-11465.

Tsuji, Y., H. Ayaki, S. P. Whitman, C. S. Morrow, S. V. Torti and F. M. Torti (2000). "Coordinate transcriptional and translational regulation of ferritin in response to oxidative stress." Mol Cell Biol **20**(16): 5818-5827.

Tuder, R. M., L. Zhen, C. Y. Cho, L. Taraseviciene-Stewart, Y. Kasahara, D. Salvemini, N. F. Voelkel and S. C. Flores (2003). "Oxidative stress and apoptosis interact and cause emphysema due to vascular endothelial growth factor receptor blockade." Am J Respir Cell Mol Biol **29**(1): 88-97.

Valavanidis, A., T. Vlachogianni and K. Fiotakis (2009). "Tobacco smoke: involvement of reactive oxygen species and stable free radicals in mechanisms of oxidative damage, carcinogenesis and synergistic effects with other respirable particles." Int J Environ Res Public Health **6**(2): 445-462.

Valko, M., D. Leibfritz, J. Moncol, M. T. Cronin, M. Mazur and J. Telser (2007). "Free radicals and antioxidants in normal physiological functions and human disease." Int J Biochem Cell Biol **39**(1): 44-84.

Valko, M., C. J. Rhodes, J. Moncol, M. Izakovic and M. Mazur (2006). "Free radicals, metals and antioxidants in oxidative stress-induced cancer." Chem Biol Interact **160**(1): 1-40.

Vandivier, R. W., P. M. Henson and I. S. Douglas (2006). "Burying the dead: the impact of failed apoptotic cell removal (efferocytosis) on chronic inflammatory lung disease." Chest **129**(6): 1673-1682.

Villeneuve, N. F., A. Lau and D. D. Zhang (2010). "Regulation of the Nrf2-Keap1 antioxidant response by the ubiquitin proteasome system: an insight into cullin-ring ubiquitin ligases." Antioxid Redox Signal **13**(11): 1699-1712.

Vinson, C. R., P. B. Sigler and S. L. McKnight (1989). "Scissors-grip model for DNA recognition by a family of leucine zipper proteins." Science **246**(4932): 911-916.

Waak, J., S. S. Weber, K. Gorner, C. Schall, H. Ichijo, T. Stehle and P. J. Kahle (2009). "Oxidizable residues mediating protein stability and cytoprotective interaction of DJ-1 with apoptosis signal-regulating kinase 1." J Biol Chem **284**(21): 14245-14257.

Wada, H., K. Kito, L. S. Caskey, E. T. Yeh and T. Kamitani (1998). "Cleavage of the C-terminus of NEDD8 by UCH-L3." Biochem Biophys Res Commun **251**(3): 688-692.

Wada, H., E. T. Yeh and T. Kamitani (2000). "A dominant-negative UBC12 mutant sequesters NEDD8 and inhibits NEDD8 conjugation in vivo." J Biol Chem **275**(22): 17008-17015.

Walden, H., M. S. Podgorski, D. T. Huang, D. W. Miller, R. J. Howard, D. L. Minor, Jr., J. M. Holton and B. A. Schulman (2003). "The structure of the APPBP1-UBA3-NEDD8-ATP complex reveals the basis for selective ubiquitin-like protein activation by an E1." Mol Cell **12**(6): 1427-1437.

Wang, C. D., N. Chen, L. Huang, J. R. Wang, Z. Y. Chen, Y. M. Jiang, Y. Z. He and Y. L. Ji (2015). "Impact of CYP1A1 Polymorphisms on Susceptibility to Chronic Obstructive Pulmonary Disease: A Meta-Analysis." Biomed Res Int **2015**: 942958.

Wang, H. and W. Gao (2016). "DJ-1 Expression in Cervical Carcinoma and its Effects on Cell Viability and Apoptosis." Med Sci Monit **22**: 2943-2949.

Wang, H., K. Liu, M. Geng, P. Gao, X. Wu, Y. Hai, Y. Li, Y. Li, L. Luo, J. D. Hayes, X. J. Wang and X. Tang (2013). "RXRalpha inhibits the NRF2-ARE signaling pathway through a direct interaction with the Neh7 domain of NRF2." Cancer Res **73**(10): 3097-3108.

Waragai, M., J. Wei, M. Fujita, M. Nakai, G. J. Ho, E. Masliah, H. Akatsu, T. Yamada and M. Hashimoto (2006). "Increased level of DJ-1 in the cerebrospinal fluids of sporadic Parkinson's disease." Biochem Biophys Res Commun **345**(3): 967-972.

Webb, D. R., R. W. Hyde, R. H. Schwartz, W. J. Hall, J. J. Condemi and P. L. Townes (1973). "Serum alpha 1-antitrypsin variants. Prevalence and clinical spirometry." Am Rev Respir Dis **108**(4): 918-925.

Wee, S., R. K. Geyer, T. Toda and D. A. Wolf (2005). "CSN facilitates Cullin-RING ubiquitin ligase function by counteracting autocatalytic adapter instability." Nat Cell Biol **7**(4): 387-391.

Wei, N., D. A. Chamovitz and X. W. Deng (1994). "Arabidopsis COP9 is a component of a novel signaling complex mediating light control of development." Cell **78**(1): 117-124.

Wills, E. D. (1966). "Mechanisms of lipid peroxide formation in animal tissues." Biochem J **99**(3): 667-676.

Wilson, M. A., J. L. Collins, Y. Hod, D. Ringe and G. A. Petsko (2003). "The 1.1-A resolution crystal structure of DJ-1, the protein mutated in autosomal recessive early onset Parkinson's disease." Proc Natl Acad Sci U S A **100**(16): 9256-9261.

Wistow, G. J., M. P. Shaughnessy, D. C. Lee, J. Hodin and P. S. Zelenka (1993). "A macrophage migration inhibitory factor is expressed in the differentiating cells of the eye lens." Proc Natl Acad Sci U S A **90**(4): 1272-1275.

Wright, J. L., H. Tai, J. Dai and A. Churg (2002). "Cigarette smoke induces rapid changes in gene expression in pulmonary arteries." Lab Invest **82**(10): 1391-1398.

Xirodimas, D. P., M. K. Saville, J. C. Bourdon, R. T. Hay and D. P. Lane (2004). "Mdm2-mediated NEDD8 conjugation of p53 inhibits its transcriptional activity." Cell **118**(1): 83-97.

Yamamoto, T., T. Suzuki, A. Kobayashi, J. Wakabayashi, J. Maher, H. Motohashi and M. Yamamoto (2008). "Physiological significance of reactive cysteine residues of Keap1 in determining Nrf2 activity." Mol Cell Biol **28**(8): 2758-2770.

- Yan, Y. F., W. J. Yang, Q. Xu, H. P. Chen, X. S. Huang, L. Y. Qiu, Z. P. Liao and Q. R. Huang (2015). "DJ-1 upregulates anti-oxidant enzymes and attenuates hypoxia/re-oxygenation-induced oxidative stress by activation of the nuclear factor erythroid 2-like 2 signaling pathway." Mol Med Rep **12**(3): 4734-4742.
- Yanagida, T., Y. Kitamura, K. Yamane, K. Takahashi, K. Takata, D. Yanagisawa, H. Yasui, T. Taniguchi, T. Taira, T. Honda and H. Ariga (2009). "Protection against oxidative stress-induced neurodegeneration by a modulator for DJ-1, the wild-type of familial Parkinson's disease-linked PARK7." J Pharmacol Sci **109**(3): 463-468.
- Yanagida, T., J. Tsushima, Y. Kitamura, D. Yanagisawa, K. Takata, T. Shibaike, A. Yamamoto, T. Taniguchi, H. Yasui, T. Taira, S. Morikawa, T. Inubushi, I. Tooyama and H. Ariga (2009). "Oxidative stress induction of DJ-1 protein in reactive astrocytes scavenges free radicals and reduces cell injury." Oxid Med Cell Longev **2**(1): 36-42.
- Yang, Y., S. Gehrke, M. E. Haque, Y. Imai, J. Kosek, L. Yang, M. F. Beal, I. Nishimura, K. Wakamatsu, S. Ito, R. Takahashi and B. Lu (2005). "Inactivation of Drosophila DJ-1 leads to impairments of oxidative stress response and phosphatidylinositol 3-kinase/Akt signaling." Proc Natl Acad Sci U S A **102**(38): 13670-13675.
- Yogosawa, S., Y. Makino, T. Yoshida, T. Kishimoto, M. Muramatsu and T. Tamura (1996). "Molecular cloning of a novel 120-kDa TBP-interacting protein." Biochem Biophys Res Commun **229**(2): 612-617.
- Yokohori, N., K. Aoshiba, A. Nagai and J. Respiratory Failure Research Group in (2004). "Increased levels of cell death and proliferation in alveolar wall cells in patients with pulmonary emphysema." Chest **125**(2): 626-632.
- Young, I. S. and J. V. Woodside (2001). "Antioxidants in health and disease." J Clin Pathol **54**(3): 176-186.
- Yu, S., T. O. Khor, K. L. Cheung, W. Li, T. Y. Wu, Y. Huang, B. A. Foster, Y. W. Kan and A. N. Kong (2010). "Nrf2 expression is regulated by epigenetic mechanisms in prostate cancer of TRAMP mice." PLoS One **5**(1): e8579.
- Zeng, G., B. Sun and N. Zhong (2012). "Non-smoking-related chronic obstructive pulmonary disease: a neglected entity?" Respirology **17**(6): 908-912.
- Zhang, D. D. and M. Hannink (2003). "Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress." Mol Cell Biol **23**(22): 8137-8151.
- Zhang, D. D., S. C. Lo, J. V. Cross, D. J. Templeton and M. Hannink (2004). "Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex." Mol Cell Biol **24**(24): 10941-10953.
- Zhang, J., T. Hosoya, A. Maruyama, K. Nishikawa, J. M. Maher, T. Ohta, H. Motohashi, A. Fukamizu, S. Shibahara, K. Itoh and M. Yamamoto (2007). "Nrf2 Neh5 domain is

differentially utilized in the transactivation of cytoprotective genes." Biochem J **404**(3): 459-466.

Zhang, J., T. Ohta, A. Maruyama, T. Hosoya, K. Nishikawa, J. M. Maher, S. Shibahara, K. Itoh and M. Yamamoto (2006). "BRG1 interacts with Nrf2 to selectively mediate HO-1 induction in response to oxidative stress." Mol Cell Biol **26**(21): 7942-7952.

Zhao, H., S. Eguchi, A. Alam and D. Ma (2017). "The role of nuclear factor-erythroid 2 related factor 2 (Nrf-2) in the protection against lung injury." Am J Physiol Lung Cell Mol Physiol **312**(2): L155-L162.

Zheng, J., X. Yang, J. M. Harrell, S. Ryzhikov, E. H. Shim, K. Lykke-Andersen, N. Wei, H. Sun, R. Kobayashi and H. Zhang (2002). "CAND1 binds to unneddylated CUL1 and regulates the formation of SCF ubiquitin E3 ligase complex." Mol Cell **10**(6): 1519-1526.

Zheng, N., B. A. Schulman, L. Song, J. J. Miller, P. D. Jeffrey, P. Wang, C. Chu, D. M. Koepp, S. J. Elledge, M. Pagano, R. C. Conaway, J. W. Conaway, J. W. Harper and N. P. Pavletich (2002). "Structure of the Cul1-Rbx1-Skp1-F boxSkp2 SCF ubiquitin ligase complex." Nature **416**(6882): 703-709.

Zipper, L. M. and R. T. Mulcahy (2002). "The Keap1 BTB/POZ dimerization function is required to sequester Nrf2 in cytoplasm." J Biol Chem **277**(39): 36544-36552.

Zuo, W., F. Huang, Y. J. Chiang, M. Li, J. Du, Y. Ding, T. Zhang, H. W. Lee, L. S. Jeong, Y. Chen, H. Deng, X. H. Feng, S. Luo, C. Gao and Y. G. Chen (2013). "c-Cbl-mediated neddylation antagonizes ubiquitination and degradation of the TGF-beta type II receptor." Mol Cell **49**(3): 499-510.

Curriculum Vitae

MEENA M. ALADDIN

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EDUCATION

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD 2012-Present
PhD, Environmental Health Science, Molecular and Translational Toxicology, Expected Graduation:
August 2017

Thesis: *Effects of MIF on Antioxidant Responses During Cigarette Smoke-Induced Oxidative Stress*

New York University-Graduate School of Arts & Science, New York, NY 2010-2012
M.Sc., Environmental Health Sciences, Molecular Toxicology Track

Thesis: *Effects of Calcite on Bioavailable Iron in Inhalable Coal Dust and Implications for Coal Workers' Pneumoconiosis*

New Jersey Institute of Technology, Newark, NJ 2006-2010

B.A. Biology and Environmental Science (double major) with concentration in Biocomplexity

Minor: Business

Magna Cum Laude

RESEARCH EXPERIENCE

PhD Candidate Sept. 2012-Present

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD

- Research focused on identifying novel pathways involved in the development of COPD

Graduate Assistant Sept. 2010-May 2012

Research Assistant Sept. 2009-Aug. 2010

New York University School of Medicine, New York, NY

- Utilized *in vivo* and *in vitro* models to study the effects of calcite on bioavailable iron in inhalable coal dust to provide novel insights into pathobiology of coal workers' pneumoconiosis
- Studies focused on the effects of concentrated air particulate matter and nickel on the cardiopulmonary system

Summer Research Intern Jun. 2008-Aug. 2008

Summer Research Intern Jun. 2007-Aug. 2007

UMDNJ-New Jersey Medical School, Dept. of Family Medicine, Newark, NJ

- Assessed the validity of the Edmonton Symptoms Assessment Scale (ESAS) at Hoboken University Medical Center on patients receiving palliative care
 - Assessed the efficacy of several patient questionnaires, including the Patient Health Questionnaire-9 (PHQ-9), as well as the Edmonton Symptoms Assessment Scale in the prediction of pain of patient receiving palliative care
-

EMPLOYMENT EXPERIENCE

Toxicologist

Apr 2017-Present

Consortium for Environmental Risk Management (CERM) Evansville, IN

- Assist clients in navigating through chemical regulatory processes by merging training in toxicology and risk assessment to characterize the human health hazard of chemicals in industrial products

Adjunct Professor

Notre Dame of Maryland University, Biology Department, Baltimore, MD Jan. 2016-Mar. 2016

Passaic County Community College, Wanaque, NJ Jan. 2012-May 2012

- Developed lesson plans and strategies to successfully facilitate teaching of difficult molecular biology concepts to students of diverse non-scientific academic backgrounds
- Led a coordinated effort with chair of the science department to develop and implement new environmental science curriculum for non-science majors
- Provided extracurricular mentoring for students interested in pursuing environmental science research

Teaching Assistant

Mar. 2014-May 2014

Bloomberg School of Public Health, Environmental Health Engineering Dept., Baltimore, MD

- Guided graduate students and assisted in preparing the course material and lectures for a course in immunotoxicology

CERTIFICATE

- Risk Science and Public Policy, Johns Hopkins Bloomberg School of Public Health, May 2016
-

ADDITIONAL SKILLS & TECHNIQUES

- **Additional Language:** Arabic (fluent)
 - **Technical Lab Skills:**
 - Mammalian cell culture (Primary and Immortalized cell lines)
 - Proteomic: Western blot; ELISA
 - Isolation of RNA and transcriptional analysis by RT-PCR
 - DNA plasmid isolation
 - Transfections (siRNA knockdown; DNA)
 - **Innovation/Business Interest:** Active interest in business development; co-development of activewear to address athletic clothing options allowing for observance of modesty
-

PUBLICATIONS & PRESENTATIONS

Aladdin, M, J Fallica, B Kim, L Serebreni, L Boyer & RL Damico. The Role of MIF on Antioxidant Responses During Cigarette Smoke Exposure. Annual Society of Toxicology Meeting. Baltimore, MD, March 2017.

Aladdin, M, J Fallica, P Hassoun, RL Damico. The Effects of Macrophage Migration Inhibitory Factor (MIF) on Antioxidants Responses to During Cigarette Smoke Exposure. American Thoracic Society Meeting. San Francisco, CA, May 2016.

Fallica, J, **Aladdin M**, RL Damico. Macrophage Migration Inhibitory Factor (MIF) is a Novel Inhibitor of ASK1-p38-XOR-Dependent Cigarette Smoke Induced Apoptosis. American Thoracic Society Meeting, Denver, CO. May 2015.

Aladdin M, E Abston, A Bucek, DL Fairweather. The Effects of Ozone Exposure on Inflammasome Activation and Heart Failure During CVB3 Myocarditis. Annual Society of Toxicology (SOT) Meeting, Phoenix, AZ, Mar. 2014.

Aladdin M, J Jian, Q Yang, LC Chen, Finkelman, R, X Huang. Laboratory Studies of the Impact of Calcite on In Vitro and In Vivo Effects of Coal Dust: A Potential Preventive Agent for Coal Workers' Pneumoconiosis. Am J Ind Med 2013 Mar; 56(3):292-9.

Aladdin M, Xi Huang, Jinlong Jian, Qing Yang, Lung-Chi Chen. Calcite as a Preventive Agent for Coal Workers' Pneumoconiosis. National Occupational Research Agenda (NORA) Symposium, Cincinnati, OH.

EXTRACURRICULAR ACTIVITIES

- Long Distance Cycling; Marathon Running/Ultrarunning: completed marathons both domestically and internationally
- Volunteer emergency medical technician/Vehicle extrication certified